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Mechanisms underlying GLI mediated transcriptional regulation

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Dedication

To my family

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Mechanisms underlying GLI mediated transcriptional regulation

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The Hedgehog signaling pathway is an evolutionarily conserved pathway that plays critical roles in both embryogenesis and adult tissue homeostasis. It has been extensively studied in fruit fly, zebra fish and mouse models. Dysregulation of Hedgehog signaling causes a wide range of birth defects, such as craniofacial defects, limb defects, and holoprosencephaly. Ectopic activation of Hedgehog signaling in adult tissues also gives rise to tumors, such as basal cell carcinoma in the skin and certain types of brain cancers.

Precise control of gene transcription is regulated by both genomic cis-regulatory elements as well as protein co-factors. To understand how Hedgehog signaling mediates transcriptional regulation, I investigated both genomic cis-regulatory elements as well as protein co-factors. Using mouse genetic approaches, I characterized a GLI responsive cis-regulatory module, GRE1, and its role in *Gremlin* transcription regulation during limb development. I demonstrate that temporal and spatial controls of key developmental genes are mediated through comprehensive co-regulation of multiple cis-regulatory modules. In the second part of my study, I purified a GLI protein complex from embryonic stem (ES) cells and identified NANOG as a novel GLI-associated protein co-factor in ES cells. Further studies revealed that NANOG inhibits Hedgehog signaling-

mediated transcription by interfering with GLI activities, providing a new mechanism of how NANOG protects ES cells from extracellular differentiation signals. I also applied and optimized the affinity-purification and mass spectrometry technique to small embryonic tissue samples, which would allow us to identify protein complexes in more relevant context.

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Chapter 1: Introduction

The Hedgehog signaling pathway is an evolutionarily conserved pathway that plays critical roles in both embryogenesis and adult tissue homeostasis. It has been extensively studied in fruit fly, zebra fish and mouse models (reviewed in Ingham et al., 2011). Dysregulation of Hedgehog signaling causes a wide range of birth defects, such as craniofacial defects, limb defects, and holoprosencephaly, while ectopic activation of Hedgehog signaling in adult tissues can also give rise to tumors, such as basal cell carcinoma in the skin and certain types of brain cancers (reviewed in Briscoe and Therond, 2013).

1.1 HEDGEHOG SIGNALING TRANSDUCTION

During embryogenesis, Hedgehog is expressed in different organizing centers of several embryonic tissues and acts as a long-range morphogen to control cell patterning and differentiation. The cellular signaling transduction of Hedgehog signaling pathway is initiated by the Hedgehog ligand binding to its receptor, Patched (Ptch), a cell-surface transmembrane protein that constitutively represses Hedgehog signaling. Upon Hedgehog binding, Ptch is no longer able to repress Smoothened (Smo), a member of the G protein-coupled receptor (GPCR) superfamily. This de-repression causes a series of intracellular signaling cascades and eventually results in the activation of the downstream transcriptional effectors of the Hedgehog pathway.

In *Drosophila,* Hedgehog signaling ultimately acts through Cubitus interruptus (Ci) on the transcriptional level. Ci is a bifunctional transcription factor that can act as either a transcription activator or transcription repressor. In vertebrates, Hedgehog signaling is mediated by the GLI transcription factors (GLI1, GLI2, and GLI3). GLI1 acts

only as a transcription activator, whereas GLI2 and GLI3 are bifunctional factors similar to the *Drosophila* Ci protein. In the absence of Hedgehog signaling, GLI2 and GLI3 are processed into transcription repressors while in the presence of Hedgehog, they are processed to be transcription activators. *Gli1* is one of the Hedgehog direct target genes and requires Hedgehog signaling to be transcribed. GLI2 and GLI3 proteins are the first responders to Hedgehog signaling. After being processed into transcription activators upon Hedgehog signaling activation, GLI2 and GLI3 turn on the expression of GLI1, which acts as a signaling amplifier to the pathway (reviewed in Briscoe and Therond, 2013).

1.2 ROLE OF PRIMARY CILIA IN HEDGEHOG SIGNALING

In vertebrates, the reception and initiation of Hedgehog signaling occurs in the primary cilium (reviewed in Goetz and Anderson, 2010). The receptor Ptch1 is found enriched in and around the primary cilium (Rohatgi et al., 2007). In the absence of Hedgehog signaling, GLI precursors are sequestered by SUFU at the base of cilia and further phosphorylated by PKA, CKI and Gsk3β. (Bariz et al., 2010; Fumoto et al., 2006; Tuson et al., 2011). This leads to proteolytic cleavage of the full-length of GLI precursors and the formation of GLI transcription repressors (GLI-R). Alternatively, in the presence of Hedgehog signaling, Ptch1 is removed from the primary cilia by unknown mechanisms, which causes the de-repression of Smo from Ptch1 repression (Kovacs et al., 2008). The resulting activation of Smo results in the dissociation of full-length GLI from SUFU and the transportation of GLI precursors from the cilia to the nucleus. After nuclear transport, GLI precursors are further phosphorylated by unknown kinase(s) to generate GLI transcription activators (GLI-A) (Humke et al., 2010). A recent study also

demonstrates that the phosphorylation levels of GLI proteins determine their activation levels. (Niewiadomski et al., 2014). This could partially explain how incremental changes in Hedgehog signaling are translated into gradient increments in transcriptional activation.

1.3 SONIC HEDGEHOG SIGNALING IN VERTEBRATE LIMB DEVELOPMENT

In vertebrates, there are three homologs of Hedgehog: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). They all serve evolutionarily conserved roles in body organization (reviewed in Varjosalo and Taipale, 2008). Among them, Shh is the best-studied Hedgehog ligand and its functions in regulating organ patterning have been extensively explored in vertebrate limb development. During limb development, Shh plays essential roles in both limb outgrowth and digit patterning. $Shh^{-/-}$ mice lose all posterior structures in the autopod (distal limb) leaving only a single digit (digit 1) and the anterior part of zeugopod (Chiang et al., 2001; Kraus et al. 2001). In contrast, ectopic expression of Shh in the limb causes ectopic growth in the anterior-posterior (AP) axis of the limb and results in the formation of polydactyly (Yang et al. 1997). This suggests that Shh signaling directly regulates formation of the posterior limb elements, including digits 2 through 5, but not the anterior elements, such as digit 1. However, a recent study demonstrates that Shh signaling also contributes to the formation of anterior elements by negatively regulating *Irx3* and *Irx5* (Li et al., 2014a).

1.3.1 ZPA-Shh signaling

During limb development, Shh is expressed in the Zone of Polarizing Activity (ZPA), which is located in the posterior limb (reviewed in Zeller et al., 2009; Rabinowitz

and Vokes, 2012). Activation of Shh in the ZPA is regulated by a number of transcription factors. For example, *Hand2* and *5' Hox* genes are required for Shh expression (Capellini et al., 2006; Trachini et al., 2006) and Tbx, Fgf8 and retinoic acid (RA) signaling are critical for restricting the Shh expression domain (Lewandoski, 2000; Niederreither et al., 2002; Rallis et al., 2005). The resulting Shh expression pattern is a gradient with high expression in the posterior of limb and low expression in the anterior. This Shh protein gradient results in inverse GLI-A and GLI-R gradients along the AP axis, which controls the expression of distinct Shh target genes. Of the three GLI proteins, GLI3 is the predominant transcriptional repressor during limb development. Compared with $Shh^{-/-}$ embryos, $Shh^{-/-};Gli3^{-/-}$ embryos have a substantial rescue in limb growth and digit formation. The expression of many genes that are lost in $Shh^{-/-}$ limb buds are restored in $Shh^{-/-}; Gli3^{-/-}$ embryos but with symmetrical gene expression patterns along the anterior-posterior axis (reviewed in Rabinowitz and Vokes, 2012).

1.3.2 Shh-FGF feedback loop

The apical ectodermal ridge (AER) is another signaling center during limb development that controls proximal-distal (PD) axis limb outgrowth. It consists of a group of specialized epithelium located at the distal limb tip. The initiation of AER requires FGF10 signaling from the nascent limb mesenchyme. After AER initiation, FGF8 start being expressed in the AER progenitors and a growth-promoting positive feedback loop forms between AER-FGF8 and mesenchymal FGF10. Then *Fgf4*, *Fgf9* and *Fgf19* are activated subsequently in the posterior AER (Lewandoski et al., 2000; Mariani et al., 2008; Sun et al., 2009). Removal of the AER results in severe limb truncations (Dudley et al. 2002; Rowe et al. 1982), and the application of FGF proteins to

limbs after AER has been removed can largely restore limbs outgrowth (Fallon et al., 1994; Niswander et al., 1993). However, of the four *Fgf* genes, *Fgf8* plays the most important role during limb development as limb buds that lack *Fgf8* are smaller and also lose certain skeletal elements (Lewandoski et al. 2000).

Shh signaling and FGF signaling form a positive feedback loop in the developing limb. Shh signaling in the ZPA is maintained by FGF signaling from the AER, which is necessary for normal limb development (Laufer et al., 1994; Niswander et al., 1994). Depletion of FGF4 and FGF8 in the AER results in a loss of Shh expression in ZPA (Boulet et al. 2004; Sun et al. 2002). Meanwhile, Shh also maintains FGF signaling by inhibiting the activity of bone morphogenetic proteins (BMPs), which would otherwise disrupt AER integrity (Zuniga et al. 1999). Specifically, Shh represses BMP activity by upregulating Gremlin, a BMP antagonist protein. Gremlin is expressed in the limb mesenchyme between ZPA and AER, and relays the Shh signaling from the ZPA to the AER to reinforce the Shh-FGF positive feedback loop (Benazet et al., 2009; Nissim et al., 2006; Panman et al. 2006; Zuniga et al., 1999).

The termination of Shh-FGF feedback loop has been extensively studied in both chicken and mice models. Study in the chicken shows that the expansion of former Shh expressing cells in the limb eventually terminates the Shh-FGF feedback loop (Scherz et al. 2004). It is found that the Shh expressing cells and their descendents can't express *Gremlin*. As the developing limb grows, the distance between the *Gremlin* expression domain and the ZPA increases, resulting in a breakdown of the Shh-FGF feedback loop. However, study in mouse reports that high levels of FGF can inhibit *Gremlin* expression (Verheyden and Sun, 2008). So the increasing FGF signaling from AER progressively increases the distance between *Gremlin* expression domain and AER. Eventually,

Gremlin no longer inhibits the BMP's negative function on AER maintenance and Shh-FGF feedback loop is no longer being maintained (Verheyden and Sun, 2008).

1.4 GREMLIN IN LIMB DEVELOPMENT

Loss of Gremlin causes the formation of fewer digits because of the disruption of Shh-FGF feedback loop and the resulting up regulation of BMP levels (Benazet et al. 2009; Khokha et al. 2003). In contrast, ectopic expression of Gremlin causes increased limb growth and the formation of extra digits (Scherz et al. 2004; Norrie et al. 2014). On the transcriptional level, *Gremlin* is induced by BMP signaling and then maintained by Shh signaling (Benazet et al., 2009; Capdevila et al. 1999; Nissim et al., 2006; Panman et al., 2006). The transcriptional regulation of Gremlin in the mouse limb bud is mediated the limb deformity locus and multiple long-range cis-regulatory modules were identified in this locus (Vokes et al., 2008; Zuniga et al., 2004; Zuniga et al. 2012). It has also been reported that Tbx2 directly represses *Gremlin* in the distal regions of the posterior limb mesenchyme (Farin et al, 2013). Loss of Tbx2 in the hindlimbs causes extended Shh and FGF signaling, resulting increased limb bud size and duplication of digit 4. Conversely, overexpression of Tbx2 leads to early local termination of Shh-FGF feedback loop, generating small limbs and fewer digits at posterior (Farin et al, 2013).

1.5 GLI BINDING REGIONS AND CIS-REGULATORY MODULES

On the genomic level, positive and negative regulations of transcriptional activities are mediated through cis-regulatory modules (CRMs), either enhancers or silencers, or both (reviewed in Noonan and McCallion, 2010; Ong and Corces, 2011). These regulatory regions are position and orientation independent, and they may be

located far from the specific gene locus. The precise mechanisms by which enhancers affect promoter activity are not well established, however, the predominant view is that enhancers are able to recruit transcriptional factors, forming a chromatin loop to bring enhancers and transcriptional factors to a specific promoter. For example, experiments examining Shh expression in the limb have shown that the long-range Shh enhancer ZRS physically interacts with the Shh transcriptional start site (Amano et al., 2009).

Enhancers are thus those genomic elements that stimulate the gene transcription while repressors are able to repress the gene transcription. Recently, several studies reported that some essential genes are controlled by multiple-enhancers: the "shadow enhancers" (Frankel et al., 2010; Hobert 2010; Perry et al., 2010). In some cases, a single CRM contains both enhancers and repressors representing different signaling inputs (Murayama et al., 2004; Perissi et al., 2004).

In vertebrates, Hedgehog ligands ultimately regulate transcription by controlling the activity of GLI transcription factors, which act as context-dependent transcription activators or repressors in response to Hedgehog signaling. Evidence from a number of studies indicates that all forms of GLI transcription factors can bind the same 9 base pair motif sequence (Hallikas et al., 2006; Muller and Basler, 2000; Peterson et al., 2012). The transcriptional output of GLI target genes is influenced both by the quality of the GLI motif sequence and the presence of tissue-specific co-factors. Studies in different organisms showed that Hedgehog responsive genes expressed near the source of Hedgehog signaling are associated with high quality GLI binding sites, while genes expressed at longer range are associated with lower quality GLI binding sites (Oosterveen et al., 2012; Parker et al., 2011; Peterson et al., 2012).

In addition, the genomic regions surrounding GLI target genes often contain multiple GLI-binding regions, suggesting the possibility that multiple CRMs could

interact together to regulate gene expression (Biehs et al., 2010; Oosterveen et al., 2012; Peterson et al., 2012; Vokes et al., 2008). If these interactions exist, they could potentially result in redundant, synergistic, or dose-dependent regulation of target genes.

GLI transcriptional targets fall into two distinct groups: genes that require GLI activation for transcription (GLI activator genes), and genes that are transcribed in the absence of GLI repression (GLI de-repression genes). GLI activators could potentially play quantitative roles in regulating the expression levels of a subset of this latter class. The behavior of target genes in response to a gradient of Hedgehog signaling suggests that competition between GLI activators and repressors could drive threshold responses that restrict the boundary of GLI-activator target gene expression (Jacob and Briscoe, 2003; Ruiz i Altaba, 1997; Wang et al., 2000). Studies that have manipulated GLI expression levels in the chick neural tube support this competition model (Oosterveen et al., 2012). The mechanism by which GLI repression prevents expression of its target genes is poorly understood, but in some cases relies on interactions between GLI repressors and specific transcription factors (Oosterveen et al., 2012). Mouse neural tubes lacking the major GLI transcriptional repressor GLI3 have a relatively modest change in target gene expression boundaries with no change in ventral neural fates and more subtle changes to intermediate identities, an effect that could be due to the robustness of the neural-specific downstream regulatory network (Balaskas et al., 2012; Persson et al., 2002).

1.6 GLI-INTERACTING PROTEINS

In addition to looking for proteins involved in Hedgehog signaling transduction and GLI protein maturation, recent studies have started to elucidate the transcriptional co-

factors involved in GLI transcriptional regulation. Ski was identified as a GLI3R-interacting protein by a yeast two-hybrid screening using the N-terminal region of GLI2 or GLI3 as bait (Dai et al., 2002). Ski and its related protein Sno act as transcription corepressors and are able to directly bind to two other corepressors, N-CoR/SMRt and mSin3A (Nomura et al., 1999). Together, these three corepressors form a protein complex with histone deacetylases (HDACs), which plays important roles in transcriptional repression. Ski was found to negatively regulate the Shh-induced activation of *Gli1* through full-length GLI3 activators, and moreover, a *Ski* mutation in mice enhanced digit abnormalities caused by the *Gli3* gene mutation. Another independent study published at the same year reported that mSin3 corepressor could be recruited by nuclear SUfU and results in the inhibition of GLI-mediated transcription (Cheng and Beachy, 2002). Consistently, these two studies suggest the corepressor-HDACs complex is one of possible mechanisms underlying GLI-mediated transcriptional regulation.

In addition to the HDAC corepressor complex, the members of the chromatin remodeling protein complex SWI/SNF have also been implicated in GLI-mediated transcriptional regulation, such as Snf5 and Brg (Jagani et al., 2010; Zhan et al., 2011). Snf5 was identified as a GLI1-interacting protein in an affinity purification-mass spectrometry experiment carried out on with mouse TM3 cells (Jagani et al., 2010). Snf5 was found as a negative regulator of the Hedgehog pathway because the loss of Snf5 leads to the activation of the Hedgehog-GLI pathway. Snf5 is also known as a tumor suppressor and biallelic inactivation was found in a majority of human malignant rhadoid tumors (MRTs), which are poorly differentiated cancers arising in the kidney, brain, liver and soft tissues (Biegel et al., 1999; Sevenet et al., 1999; Versteege et al., 1998). Re-expression of Snf5 in MRT cell represses GLI1 activity and consistent with this,

Hedgehog-GLI target gene expressions were found in primary MRTs, suggesting a model that the tumor suppressor Snf5 represses Hedgehog signaling in adult tissue and loss of Snf5 causes ectopic activation of Hedgehog signaling and eventual tumorgenesis. A recent study reported that chromatin remodeling protein Brg can interact with GLI proteins and plays a dual role in regulating Hedgehog signaling during mouse neural development: it's required for both repression of basal expression and for activation of signal-induced Shh target gene transcription (Zhan et al., 2012). Interestingly, Snf5 and Brg are both tumor suppressors as well as the core subunits of the SWI-SNF complex. These studies suggest that the Hedgehog pathway is likely to be the common target of the SWI-SNF complex and is also central to the tumor suppressor activity of Snf5 and Brg.

Mediator is a large complex of proteins that serves as an interface between gene-specific transcription factors and the RNA polymerase II general transcription machinery (Conaway et al., 2005; Kornberg, 2005). It has been proposed to function as a general conduit and integrator of regulatory signals and several Mediator subunits are required for transcription activation in response to distinct cellular signaling pathways (Boyer et al., 1999; Kato et al., 2002; Lau et al., 2003). GLI3 physically and functionally interacts with the Mediator subunit MED12 (Zhou et al., 2006). GLI3 is able to bind with MED12 and Mediator/CBP complex through a transactivation domain MBD (MED12/Mediator-binding domain). Disruption of the GLI3-MED12 interaction inhibits while MED depletion enhances both MBD transactivation activity and GLI3-dependent gene induction in response to Shh signaling.

The phosphorylation state of GLI regulates its transcriptional activities. One of the possible reasons is that some phosphorylation sites affect GLI's binding ability to it co-factors. 14-3-3 has been identified as a GLI negative regulator and is able to interact with all three GLI proteins (GLI1-3) through a phosphorylation dependent manner

(Asaoka et al., 2010). The 14-3-3 interaction with GLI causes repression of GLI activator's transcription activity. More interestingly, the conserved phosphorylation site--Ser640 for GLI1, Ser956 for GLI2 and Ser1006 for GLI3, is one of the PKA substrates on GLI proteins. PKA is one of the well-studied Hedgehog signaling negative regulators. One reported mechanism by which PKA influences Hedgehog signaling involves promoting the processing of the GLI precursor into repressor form (Chen et al., 1998; Wang et al., 2000; Pan et al., 2006). However, mediating 14-3-3 and GLI interaction may be a distinct PKA-dependent mechanism involved repressing Hedgehog signaling.

1.7 HEDGEHOG SIGNALING IN STEM CELLS AND CANCER

Because of its important roles in regulating early embryo development, dysregulation of Hedgehog signaling gives rise to a wide range of birth defects, including craniofacial defects, limb defects, and holoprosencephaly. Hedgehog signaling is also important for regulating homeostasis of adult tissues, including the epithelia of many internal organs and brain (reviewed in Jiang and Hui, 2008; Petrova and Joyner, 2014). For example, Hedgehog signaling is required for the regeneration of the pulmonary epithelium (Watkins et al., 2003), prostate epithelium (Karhadkar et al. 2004), and exocrine pancreas (Fendrich et al., 2008). Moreover, Hedgehog signaling plays a regulatory role in the formation and maintenance of adult hematopoietic stem cells (HSCs) in both *Drosophila* and zebrafish (Gering and Patient, 2005; Mandal et al., 2007), as well as in neural stem cells (NSCs) (Ahn and Joyner 2005; Balordi and Fishell, 2007; Dahmane et al., 2001; Lai et al., 2003; Machold et al., 2003; Palma et al., 2005; Wechsler-Reya and Scott, 1999)

Ectopic activation of Hedgehog signaling in adult tissue gives rise to tumors. *Gli1* was initially identified because of its elevated expression in human glioma, a central nervous system tumor derived from the glial cells (Kinzler et al., 1987). Similarly, mutations in Hedgehog signaling pathway, including *Ptch1* and *Smo,* and elevated level of *Gli1* were also observed in basal cell carcinoma (Fan and Khavari, 1997; Ora et al., 1997), and certain types of brain cancers (Hahn et al., 1996; Johnson et al., 1996; Taylor et al., 2002). It is widely assumed that the "GLI code" regulated by Hedgehog signaling is one of the reasons adult stem cells/progenitor cells turn into cancer stem cells (CSCs) (reviewed in Aberger and Ruiz i Altaba, 2014). Through the GLI code, Hedgehog signaling modulated the fate and normal behavior of stem cells. Recent studies also reported high expression levels of *Nanog* and *Gli1* co-existed in different types of tumors and a NANOG-GLI positive feedback loop has been found in glioblastoma multiforme and medulloblastoma (Po et al. 2010; Zbinden et al. 2010). These results suggest that Hedgehog signaling could affect stem cells and progenitor cells bymodulating the pluripotent factor NANOG.

1.8 SUMMARY

To understand the basic mechanisms underlying Hedgehog signaling mediated transcriptional regulation, I conducted my PhD thesis work from two aspects. In the first part, my work focused on the long distance CRMs that regulate dynamic expression of *Gremlin* during limb development. By using a combination of mouse genetics and embryological manipulations, I characterized the first GLI-responsive CRM (termed GRE1, GLI responsive element 1) in vertebrates. I found GRE1 could act as both an enhancer and a silencer. As an enhancer, its activity requires sustained Hedgehog

activation; as a silencer, GRE1 prevents ectopic transcription of *Gremlin* driven through additional CRMs. In the second part of my thesis work, I searched for GLI co-factors in embryonic stem cells and embryonic limb buds. By applying affinity purification and mass spectrometry, I identified a number of potential GLI-interacting proteins from embryonic stem cells. I further explored the interactions between pluripotent factor NANOG and GLI proteins. My study suggests that NANOG represses Hedgehog signaling-mediated transcription by interfering with GLI transcription factors.

Chapter 2: Characterization of GLI-responsive cis-regulatory module GRE1[1]

The transcriptional response to the Hedgehog pathway is mediated by GLI proteins, which function as context-dependent transcriptional activators or repressors. However, the mechanism by which GLI proteins regulate their target genes is poorly understood. Here, we have performed the first genetic characterization of a GLI-dependent cis-regulatory module (CRM), focusing on its regulation of *Gremlin* in the mammalian limb bud. The CRM, termed GRE1 (GLI responsive element 1) can act as both an enhancer and a silencer. The enhancer activity requires sustained Hedgehog signaling. As a GLI-dependent silencer, GRE1 prevents ectopic transcription of *Gremlin* driven through additional CRMs. In doing so, GRE1 works with additional GREs to robustly regulate *Gremlin*. We suggest that multiple GLI CRMs may be a general mechanism for mediating a robust transcriptional response to the Hedgehog pathway.

2.1 IDENTIFICATION OF GLI RESPONSIVE ELEMENT 1 (GRE1)

In a genome-wide chromatin immunoprecipitation study, we previously identified a 438-bp GLI3 binding region located over 100kb downstream of *Gremlin* that exhibited enhancer activity in transient transgenic limb buds in a region partially overlapping with *Gremlin* gene expression (Fig 2.1A) (Vokes et al., 2008). Enhancer activity is dependent on the presence of at least one GLI motif as mutations of the motif resulted in

[1] This Chapter is adapted and modified from "Li Q, Lewandowski JP, Powell MB, Norrie JL, Cho SH, Vokes SA (2014). A Gli silencer is required for robust repression of gremlin in the vertebrate limb bud. *Development* 141:1906 -1914". Limb culture assays were performed by Jordan Lewandowski, *GRE1lacZ* transgenic analysis was performed by Marian Powell, skeleton preparations were performed by Jacqueline Norrie, GRE1 BAC deletion was generated by Seung Hee Cho.

a complete lack of enhancer activity in G0 transgenic embryos (Vokes et al., 2008). We sought to characterize GLI enhancer regulation in the context of this CRM, which is henceforth referred to as GRE1 (GLI responsive element 1). Embryos derived from three founder lines of stable transgenics had β-galactosidase activity in posterior limb bud mesenchyme in an identical domain to that previously reported for transient transgenics (Vokes et al., 2008). We selected one line, Tg(Rr26-lacZ)438Svok, henceforth referred to as *GRE1LacZ*, for further analysis. β-galactosidase activity was first detected in embryos at E10.0 (31-32 somites; Fig 2.1B), well after the reported onset of *Gremlin* expression at ~E9 (Benazet et al., 2009; Zúñiga and Zeller, 1999). The enhancer had activity in the posterior limb within a subregion of the Shh-responsive domain. *Shh* expression initiates in the limb bud around 28 somites (E9.75) (Charité et al., 2000), and the lag in reporter expression is consistent with the reported kinetics of Shh-mediated induction of *Gremlin* (Benazet et al., 2009). By E10.5, β-galactosidase activity was strongly upregulated and persisted until late E11.5. By E11.75 expression was reduced and had retreated from the distal limb mesenchyme. Expression was nearly absent by E12.0 except for faint staining in the proximal middle of the condensing digit mesenchyme (Fig 2.1C-F). No expression was detected after E12.0, correlating with the termination of Shh activity in the limb (Echelard et al., 1993; Harfe et al., 2004). Although the enhancer analyses focused on forelimb expression, we observed similar domains in the hindlimbs (Fig 2.1G).

2.2 GRE1 ENHANCER ACTIVITY REQUIRES SHH SIGNALING

To determine if GRE1 is responsive to Shh signaling, we examined enhancer activity at E10.5 in Shh gain and loss-of-function backgrounds. In contrast to wild-type or heterozygous littermates, $Shh^{-/-}$; $GRE1LacZ^{+/-}$ embryos had no detectable β-

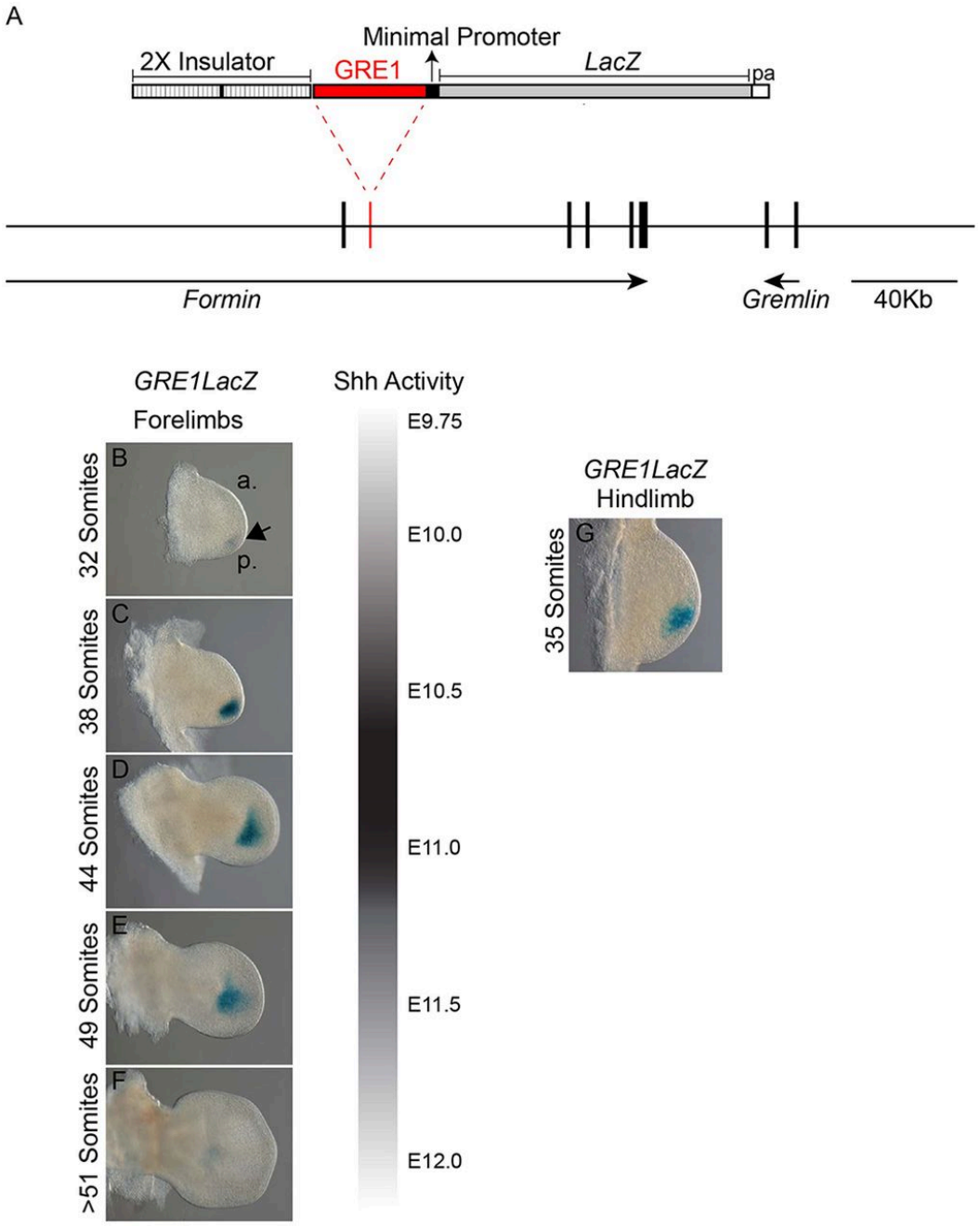

Figure 2.1 GRE1 enhancer activity correlates temporally with Shh activity in mouse

A: Schematic showing the location of GRE1 in relation to the *Gremlin* locus and GRE1LacZ transgenic construct. B-G: *GRE1LacZ*+/- forelimbs from E10 to E12 (B-F) and hindlimb from E10.5 (G) embryos stained for β-galactosidase.

galactosidase activity (6/6 embryos) and, consistent with previous studies, *Gremlin* gene expression was highly downregulated (Fig 2.2B,B')(Zúñiga and Zeller, 1999). We also examined expression by activating high levels of Hedgehog signaling throughout the limb bud using a Cre inducible dominant active allele, $Rosa^{SmoM2}$ (Jeong et al., 2004). $Prx1Cre;Rosa^{SmoM2c/+};GRE1LacZ^{+/-}$ embryos expressed both the *Gremlin* transcript and β-galactosidase activity throughout the entire distal limb bud (11/11 embryos; Fig 2.2C, C'), indicating that high levels of Hedgehog pathway activity were sufficient to activate *GRE1LacZ* along the anterior-posterior axis. *Gremlin* gene expression appeared patchy (Fig 2.2C'), and while the reason for this expression in unclear, it is consistent with observations from another study that also activated the Hedgehog pathway throughout the limb bud (Butterfield et al., 2009). Because *PrxCre* is active throughout the limb mesenchyme (Logan et al., 2002), the distal restriction of GRE1 enhancer activity suggested that additional, distal factors are also required for *Gremlin* expression. We concluded that Shh is both necessary and sufficient for enhancer activation.

2.3 GRE1 ENHANCER DOMAIN IS REGULATED BY GLI ACTIVATION

We next examined enhancer activity in $Gli3^{-/-};GRE1LacZ^{+/-}$ embryos at E10.5. Consistent with previous reports, *Gremlin* gene expression expands anteriorly in $Gli3^{-/-}$ embryos (Fig 2.2D'). In contrast the enhancer activity domain, marked by β-galactosidase staining, does not expand anteriorly (Fig 2.2D). Instead, the domain is significantly reduced in all $Gli3^{-/-}$ embryos (5/5) compared to heterozygous littermates ($p = 0.0007$). The reduction in enhancer activity suggests a role for GLI3 activator in the posterior limb. Consistent with this, $Gli3^{-/-}$ limbs at this stage had significantly reduced levels of the GLI activator target gene *Gli1* (Fig 2.3A). There was also a trend towards a

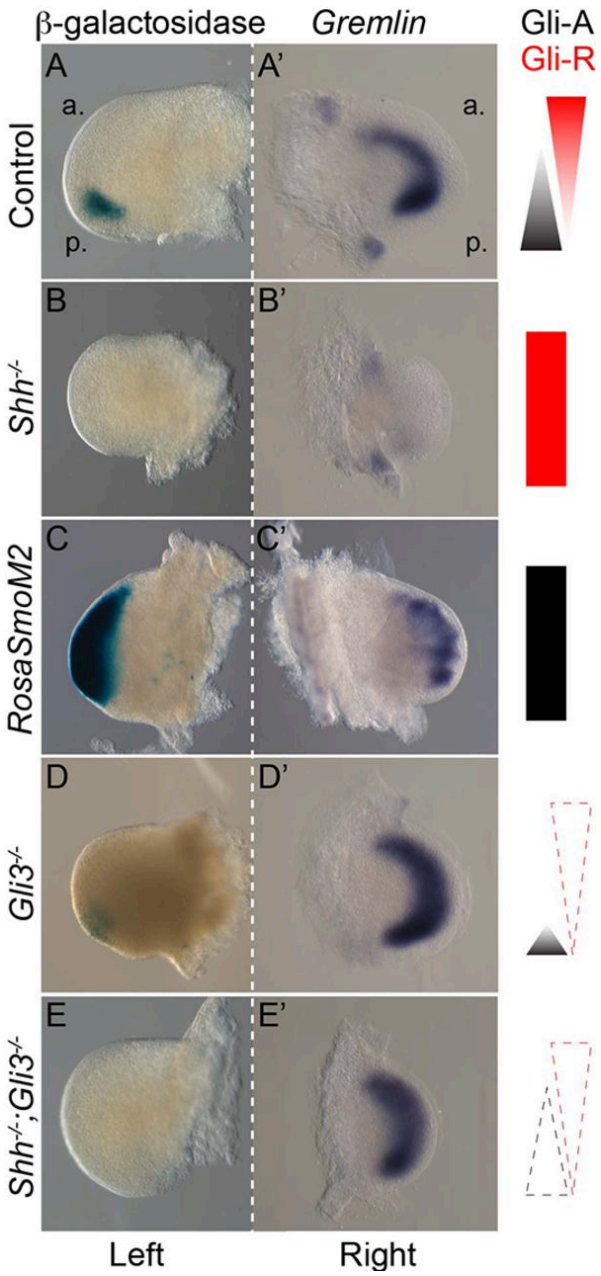

Figure 2.2 GRE1 enhancer activity requires GLI activation

A-E': E10.5 *GRELacZ* forelimbs indicating enhancer activity in various genetic backgrounds. The corresponding GLI gradient status (GLI activator in black, GLI repressor in red) is indicated to the right of each set of images. Embryos were dissected into halves; the left forelimb (left column) was stained for enhancer activity (β-galactosidase) and the corresponding right forelimb(right column) was assayed for *Gremlin* gene expression.

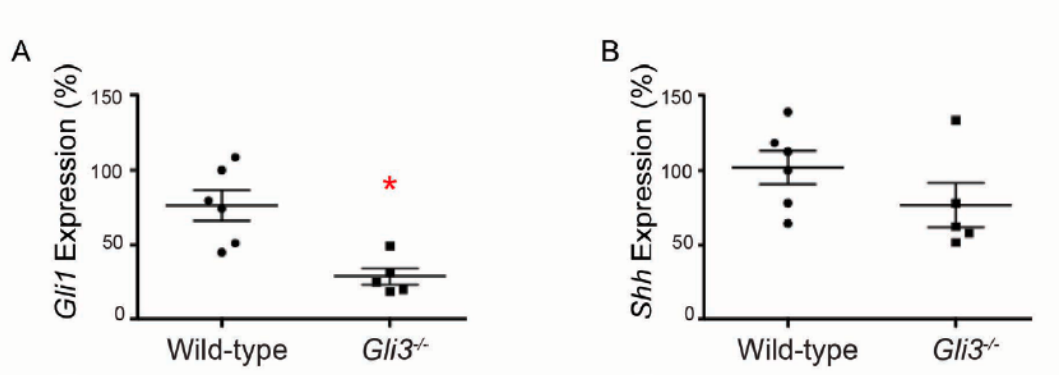

Figure 2.3 *Gli1* is significantly reduced in *Gli3*$^{-/-}$ forelimbs

Data points indicate relative gene expression assayed by qRT-PCR for pairs of forelimbs from single embryos normalized to a single 32 somite wild-type sample for wild-type (n=6) and *Gli3*$^{-/-}$ littermates (n=5). All embryos were between 32-34 somites. A: The presence of an asterisk (red) indicates that *Gli1* expression is significantly reduced in *Gli3*$^{-/-}$ forelimbs (Mann-Whitney U Test; U=1.000, p=0.0087). B: In the same sample, *Shh* levels tend to be reduced although not to statistically significant levels (Mann-Whitney U Test; U=6.000, p=0.1255). The bars indicate the mean and standard error of mean.

25% reduction in Shh levels that did not reach statistically significant levels (Fig 2.3B). These results are consistent with previous studies that have shown that *Gli3*$^{-/-}$ limb buds have reduced GLI activator levels as a combination of the direct reduction in GLI3 activator and reduced levels of Shh (Bai et al., 2004; Galli et al., 2010; Wang et al., 2007).

In *Shh*$^{-/-}$;*Gli3*$^{-/-}$;*GRE1LacZ*$^{+/-}$ embryos, *Gremlin* expression persists in limb buds in a depolarized fashion as shown previously (Fig 2.2E') (Aoto et al., 2002; Litingtung et al., 2002; te Welscher et al., 2002). However, the limb buds had an absence of β-galactosidase staining (3/3 embryos; Fig 2.2E), indicating that GLI activation is required for GRE1 enhancer activity. This is consistent with our previous work that identified a GLI motif that was essential for driving enhancer activity (Vokes et al., 2008). Although we focused on enhancer activity at E10.5, we noticed a single *Shh*$^{-/-}$;*Gli3*$^{-/-}$;*GRE1LacZ*$^{+/-}$

Figure 2.4 GRE1 enhancer activity in various genetic backgrounds at E11

GRE1LacZ$^{+/-}$ forelimbs stained for β-galactosidase activity. Limb buds in A,D,E,F are from embryos that were 40 somites, B from 38 somites, C from 42 somites. "a", anterior; "p.", posterior.

embryo at E11 that had a thin stripe of β-galactosidase activity extending in a symmetrical arc across the distal limb bud (Fig 2.4E). A similar arc was seen in the anterior of E11 *Gli3*$^{-/-}$*;GRE1LacZ* embryos while no activity was present in *Shh*$^{-/-}$ embryos (Fig 2.4). It is presently unclear whether this expression reflects a weak, late role for GLI3 repression in restricting the enhancer domain or some type of indirect activation (see Discussion).

2.4 GRE1 ENHANCER ACTIVITY REQUIRED SUSTAINED GLI ACTIVATION

To determine the time period during which GRE1 requires GLI-activator for enhancer activity, we used an established ex vivo limb bud culture assay, treating *GRE1LacZ*$^{+/-}$ limb buds with the Hedgehog pathway inhibitor cyclopamine (Panman et al., 2006). We cultured one forelimb in media containing cyclopamine while the contralateral side was cultured in control media, providing an internal control for staging and embryo variability (Fig 2.5B). As expected from the lack of activity in *Shh*$^{-/-}$ embryos (Fig 2.2B), limb buds cultured in cyclopamine at stages before enhancer activity is

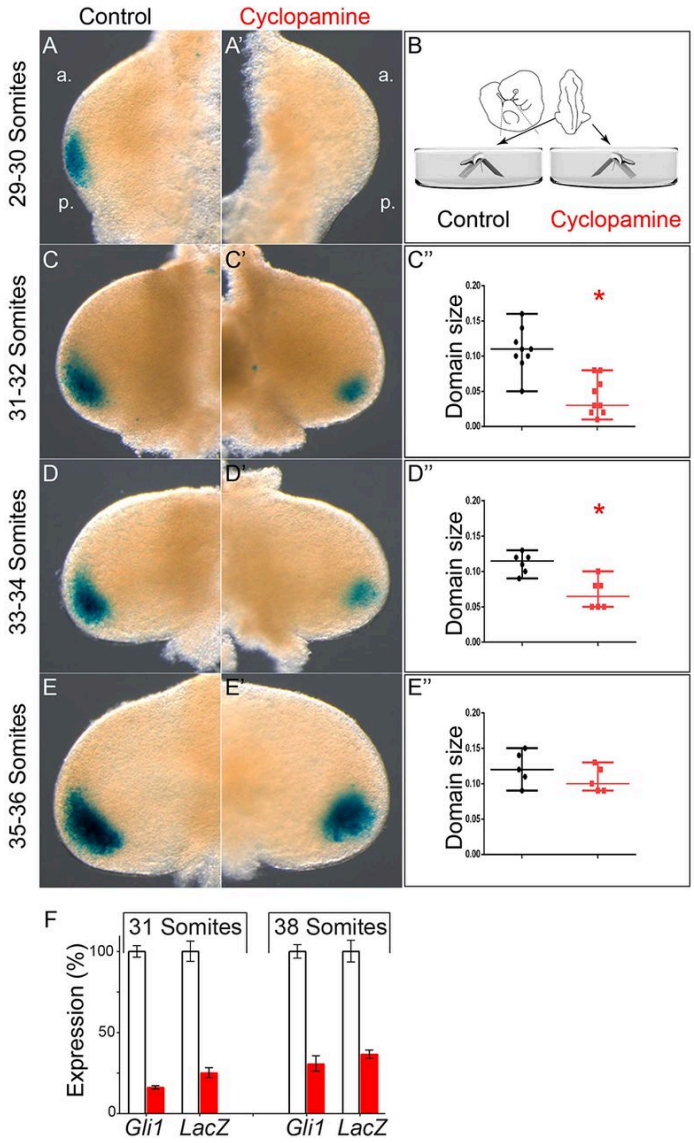

Figure 2.5 GRE1 enhancer activity requires sustained Shh signaling.

GRE1LacZ$^{+/-}$ forelimbs were cultured in vehicle-containing control media (A-E) while their contralateral forelimbs were cultured in cyclopamine (A'-E') as shown in (B). Graphs indicating the domain size measured by the ratio of the β-galactosidase stained area to the total limb bud area for control (black) or cyclopamine treated (red) limb buds. Data points indicate the median and range of values. The presence of an asterisk indicates statistically significant difference (Mann-Whitney U test). Specific values are: C'' U=3.5, p =0.0004; D'' U=1.5, p = 0.0065; E'' U=7.5, p = 0.3333. (F) Quantitative RT-PCR experiments from single forelimbs and their contralateral control limbs for representative 31 and 38 somite embryos cultured in control and cyclopamine containing media (error bars indicate the standard error of mean). 'a.', anterior; 'p.', posterior.

detected (29-30 somites) resulted in a complete loss of β-galactosidase(Fig2.4A,A'). In limb buds cultured at 31-32 somites, there is a strong reduction (61%) in the size of the enhancer activity domain when compared to the control side (Fig 2.5C-C''; p = 0.0004). Limbs cultured from 33-34 somites have more modest reductions (39%) in the size of the enhancer domain (Fig 2.5D-D''; p =0.0065). The domain size no longer depends on Shh signaling from 35-36 somites onwards (Fig 2.5E-E''; p = 0.3333). These results indicate that Shh signaling is required for expanding the domain of enhancer activity until 35-36 somites. Since residual β-galactosidase protein could persist after the cessation of transcriptional activity from the reporter, it was not possible to determine if Shh is required to maintain enhancer activity with this approach. To circumvent this problem we performed additional limb bud cultures on 32 and 38 somite embryos and measured LacZ expression by qRT-PCR. As a control to ensure that the experimental conditions resulted in robust inhibition of Shh signaling, we measured the expression of obligate Shh target gene *Gli1* (Panman et al., 2006). When forelimbs from 32 somite embryos were cultured, they had an 84% reduction in *Gli1* gene expression and a 75% reduction in LacZ expression. Similarly, forelimbs cultured from 38 somites embryos had a 70% reduction in *Gli1* and also had a 64% reduction in *LacZ* (Fig 2.5F).The change in gene expression at later stages contrasts with the stable expression domains indicated by β-galactosidase staining (Fig 2.5E-E''). We concluded that establishing the enhancer domain requires Shh signaling transiently until 35 somites, while enhancer activity within the domain continues to require sustained Shh signaling.

In addition to requiring Hedgehog signaling, GRE1 could potentially be negatively regulated by FGF signaling, which has previously been shown to negatively regulate *Gremlin* during later limb specification (Verheyden and Sun, 2008). To test this,

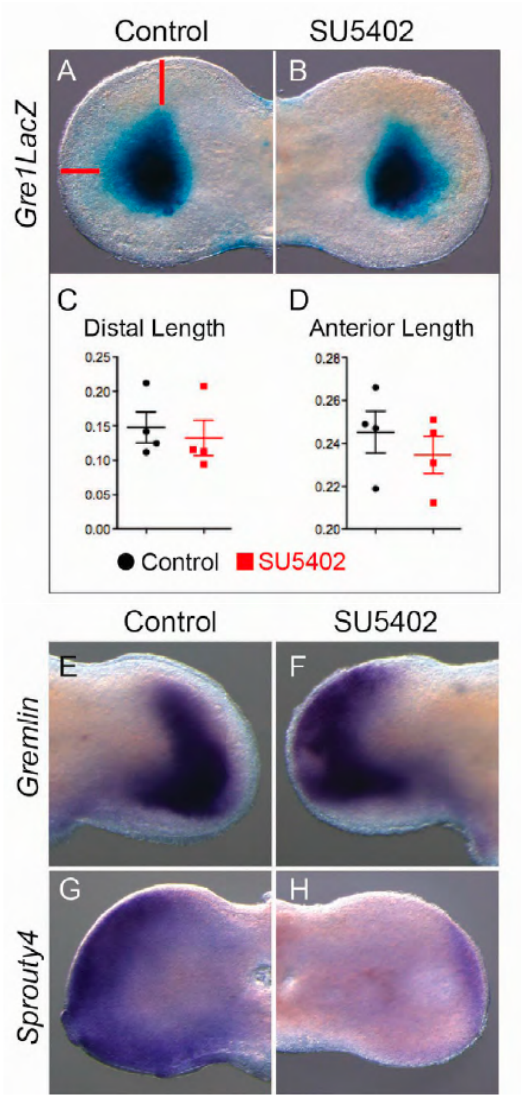

Figure 2.6 GRE1 enhancer activity is not negatively regulated by FGF

E11.5 *GRE1LacZ*$^{+/-}$ forelimbs (45-48 somites) were cultured in vehicle-containing control media (0.1% DMSO) (A) while their contralateral forelimbs were cultured in 10uM SU5402 8 hours (B) and stained for β-galactosidase activity. The normalized distance of β-galactosidase domain from the distal (C) and anterior (D) limb (schematized as red lines in (A)) is not significantly altered in SU5402-treated embryos (Mann Whitney U Test). Horizontal lines indicate the mean and standard error. There is a reduction in the FGF target gene Sprouty4 in SU5402 cultured limb buds (H) compared to contralateral limb buds cultured in control media (G). A, B, G, H are from a 48 somite embryo; E, F are from a 45 somite embryo.

we cultured additional GRE1LacZ limb buds in the presence and absence of the FGF inhibitor SU5402 (Mohammadi et al., 1997). Consistent with previous results, Gremlin was expanded in the distal-anterior limbs in SU5402 treated cultures, but GRE1LacZ activity was not inhibited by FGF signaling (Fig 2.6).

2.5 GLI REPRESSION OF THE CRM PREVENTS ECTOPIC ANTERIOR EXPRESSION OF GREMLIN

To determine the effect of GRE1 on *Gremlin* expression, we examined G0 transgenics at E11- 11.5 (41-48 somites) containing a previously generated BAC in

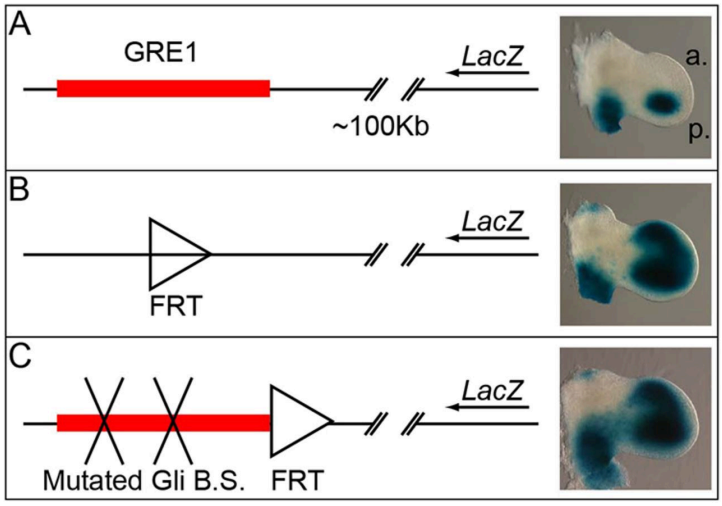

Figure 2.7 GRE1 is necessary for GLI repression of Gremlin in the anterior limb buds

A-C: BAC construct has *lacZ* inserted into the *Gremlin* coding region to act as a reporter of its transcriptional expression. Representative forelimb buds are shown from E11 G0 transgenic embryos containing the indicated BAC construct. Gremlin expression from the unaltered BAC transgene is present exclusively in the posterior limb bud (A). Deletion of GRE1 (B) or mutation of the two GLI motifs present within GRE1 (C) results in ectopic distal-anterior expression.

which *LacZ* was inserted into the *Gremlin* coding region (Zuniga et al., 2004). Consistent with previous studies, β-galactosidase activity was restricted to the posterior limb in β-galactosidase-expressing embryos (0/6 have anterior expression; Fig 2.5A) (Zuniga et al., 2004; Zuniga et al., 2012). The expression domain was similar although not identical to *GRE1LacZ* expression at the same stage (Fig 2.1D), and we hypothesized that deletion of GRE1 would reduce or eliminate reporter gene expression. Unexpectedly, the deletion resulted in ectopic anterior limb bud expression in most embryos (7/10; Fig 2.5B). The ectopic anterior expression suggested the presence of additional CRM(s) that either individually or collectively and have pan-limb enhancer activity. It also suggested that GRE1 is acting as a silencer in the anterior limb. We generated a third set of constructs in which GRE1 was re-inserted into the BAC with mutations in the two GLI DNA binding motifs present in the CRM (see methods). These forelimbs also contained ectopic anterior expression (3/3 embryos; Fig 2.5C). We concluded that GLI binding regions within GRE1 mediate silencer activity, preventing ectopic *Gremlin* transcription. This is consistent with the anterior expansion of *Gremlin* observed in *Gli3* embryos (Fig 2.2D'). Synthesizing this result with the *GRE1LacZ* enhancer activity studies above, we conclude that GRE1 can act as both an enhancer in posterior limb and as a silencer that prevents ectopic anterior expression (see Discussion, Fig 2.11).

2.6 THE GRE1 CRM FUNCTIONS AS A GLI-MEDIATED SILENCER

The results described so far suggested that the GRE1 CRM likely mediates the transcriptional activity of *Gremlin*. We then generated mice containing a deletion of GRE1. $Gremlin^{\Delta GRE1/\Delta GRE1}$ forelimbs expressed *Gremlin* and *Formin* at levels that are indistinguishable from wild-type control forelimbs (Fig 2.8A-C). $Gremlin^{\Delta GRE1/\Delta GRE1}$ mice

were viable and fertile with normal skeletal patterning (Fig 2.8D-G). Embryos containing one null allele of *Gremlin* (Khokha et al., 2003) and a second allele harboring the deletion of GRE1 also had normal skeletal patterning (Fig 2.9). These results indicate that GRE1 is not necessary for normal skeletal development. The *Gremlin* gene expression domain was nearly normal in *Gremlin*$^{\Delta GRE1/\Delta GRE1}$ embryos at E11 (Fig 2.8A,B) but at earlier stages, the distal anterior boundaries of expression were more diffuse (Fig 2.8A,B). In light of these results, we hypothesized that redundant GLI - dependent CRMs

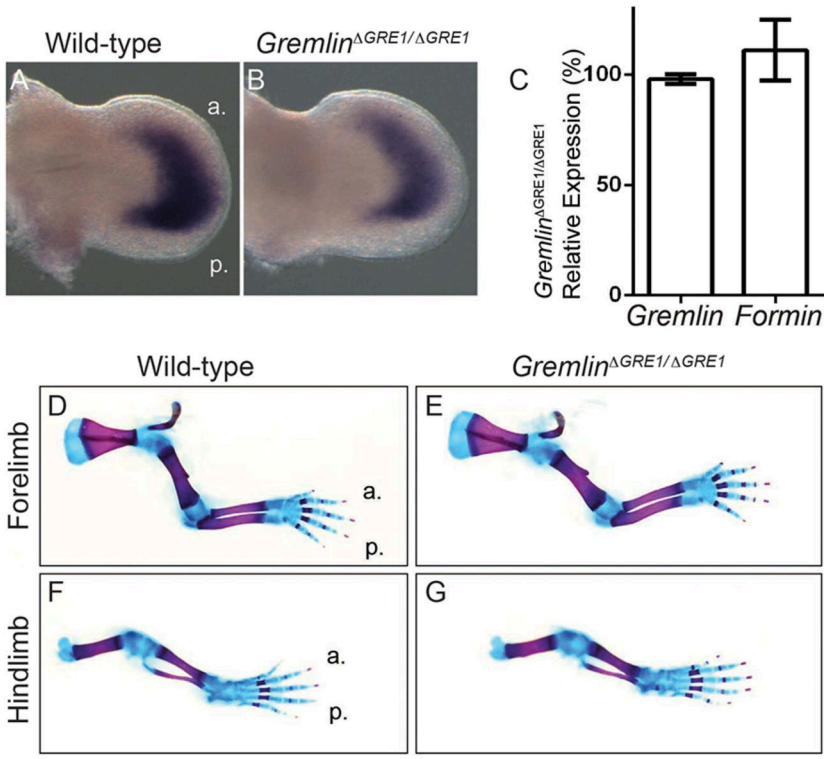

Figure 2.8 GRE1 is not essential for limb development

A, B: Gremlin expression in forelimbs at E11 (41 somites). C: Embryos express *Gremlin* and *Formin* at levels that are statistically indistinguishable from wild-type embryos (one sample t-test, two tails; *Gremlin*, P=0.1172; *Formin*, P=0.4548). Values are normalized to the distal marker jagged 1. Error bars represent the standard error of mean from four independent biological samples (E10.5, 34-37 somites). D-G: E18.5 skeletal preparations showing forelimbs and hindlimbs of the indicated genotypes. a., anterior; p., posterior.

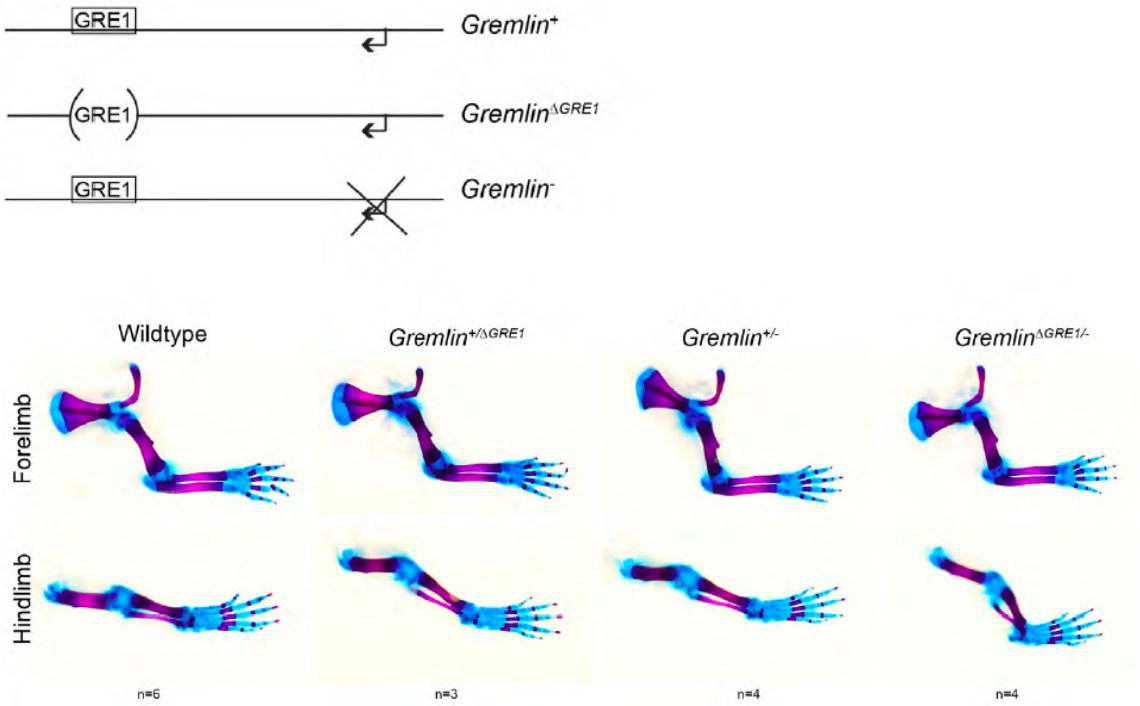

Figure 2.9 A single copy of the *Gremlin* with GRE1 is sufficient for limb development

Embryos containing a null allele of *Gremlin* and the other allele with a deletion of CRM have normal limb skeletal patterning. Images depict forelimbs and hindlimbs from the same embryo at E18.5 stained for bone (Alizarin Red) and cartilage (Alcian Blue). The numbers of skeletons that were analyzed for each genotype are indicated below.

might regulate *Gremlin*. Two additional GLI-binding regions are present within the *Gremlin* locus (Vokes et al., 2008). One of these regions was recently shown to have Shh-responsive enhancer activity and to be critical for mediating BAC reporter activity in transgenic embryos (Zuniga et al., 2012). We hypothesized that our GLI CRM might be redundant with other GLI-dependent CRMs under normal conditions but still required for robust regulation of *Gremlin*.

Studies in *Drosophila* have tested the robustness of transcriptional responses to shadow enhancers by examining CRM deletion phenotypes at the outer ranges of permissive temperatures or by removing one copy of an upstream regulator (Frankel et al.

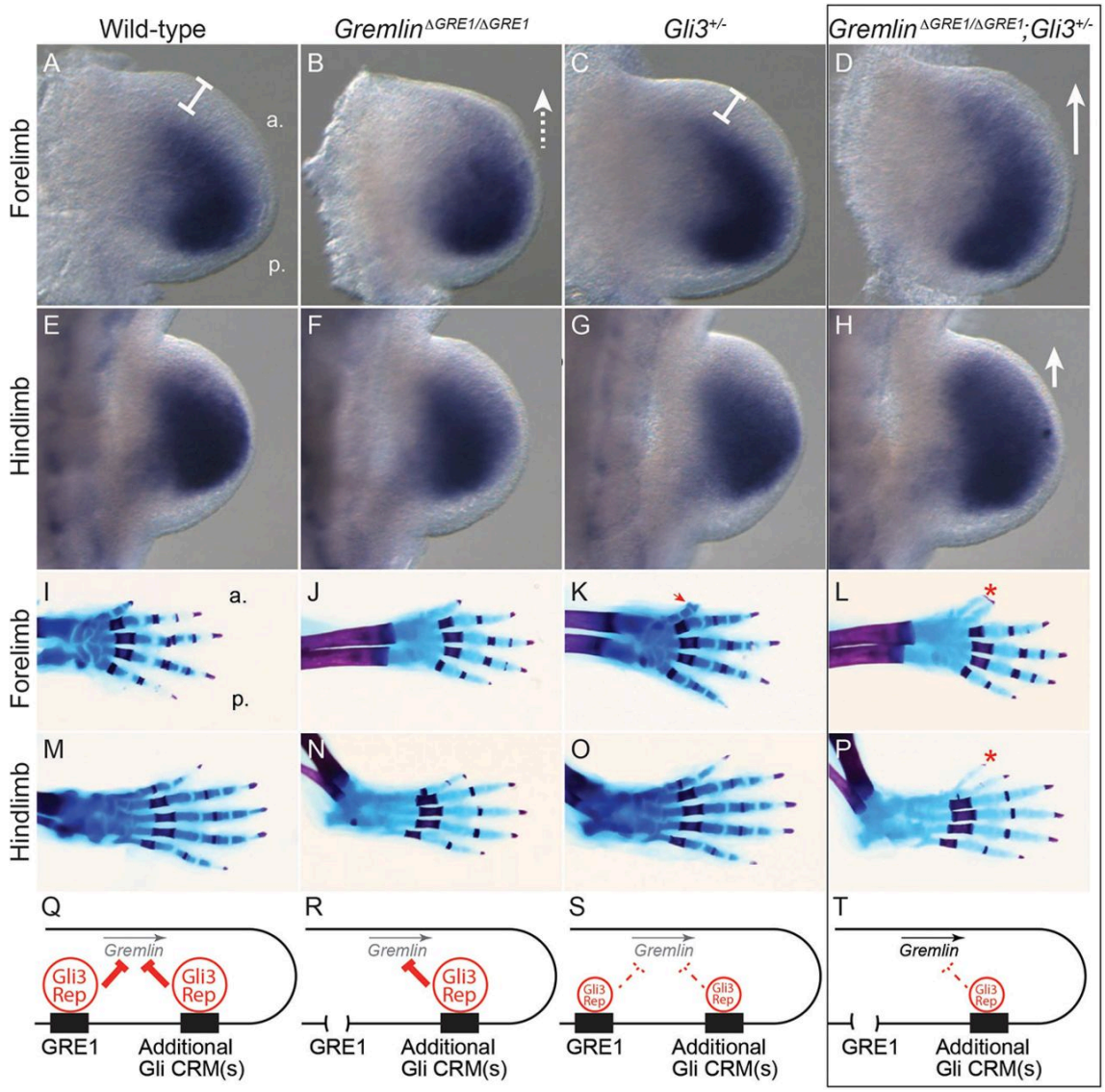

Figure 2.10 GRE1 interacts genetically with GLI3 to repress *Gremlin*

A-H: *Gremlin* expression in various genetic backgrounds in the forelimbs of 35-36 somite embryos (A-D) and hindlimbs of 37-38 somite embryos (E-H). The white brackets in panels (A,C) indicate the *Gremlin* free domain in anterior limb buds. White arrows (B,D,H) indicate ectopic distal-anterior *Gremlin* expression in *Gremlin*$^{\Delta GRE1/\Delta GRE1}$ backgrounds. (I-P) E18.5 skeletal preparations of the hand (J-L) or foot (M-P) in various genetic backgrounds. The arrowhead (K) highlights a bifurcated thumb; the asterisks (L,P) indicate polydactylous digits. (Q-T) Schematic models showing how Gli3 repression of *Gremlin* might occur in various genetic backgrounds.

2010; Perry et al., 2010). We used the latter strategy to examine *Gremlin* expression in *Gremlin*$^{\Delta GRE1/\Delta GRE1}$ embryos containing a single copy of *Gli3,* which is sufficient to prevent the distal-anterior expression of *Gremlin* seen in *Gli3*$^{-/-}$ embryos (te Welscher et al., 2002)(Fig 2.2D'). At E10.5, both wild-type and *Gli3*$^{+/-}$ littermates have a sharp boundary of *Gremlin* expression that is restricted from the most distal-anterior mesoderm in the forelimbs of all embryos (n=7; brackets in Fig 2.10A,C). *Gremlin*$^{\Delta GRE1/\Delta GRE1}$ littermates have forelimbs with less pronounced distal-anterior borders of *Gremlin* and with weak ectopic expression in the anterior limb mesoderm directly adjacent to the apical ectodermal ridge (n=10; dashed arrow in Fig 2.7B). In contrast, *Gremlin*$^{\Delta GRE1/\Delta GRE1}$;*Gli3*$^{+/-}$ littermates have forelimbs with ectopic distal-anterior *Gremlin* expression that is broader and stronger than in *Gremlin*$^{\Delta GRE1/\Delta GRE1}$ forelimbs (n=8; Fig 2.10D). This expression is significantly different from *Gli3*$^{+/-}$ ($p = 0.0002$) or *Gremlin*$^{\Delta GRE1/\Delta GRE1}$ forelimbs ($p<0.0001$), indicating a genetic interaction between GLI3 and the *Gremlin*$^{\Delta GRE1}$ allele.

An expansion of Gremlin protein into the anterior distal mesenchyme would inhibit BMPs, causing an expansion in anterior growth (Lopez-Rios et al., 2012; Pizette and Niswander, 1999). This growth would likely result in anterior polydactyly, which is also seen in mice with reduced BMP activity (Dunn et al., 1997; Selever et al., 2004). In the mixed genetic background present in our colony, the presence of the *Gli3*$^{+/-}$ 'extra toes' allele only rarely results in mice or embryos with fully polydactylous digits. In this study, all of the *Gli3*$^{+/-}$ embryos had a single nub (a fleshy outgrowth that sometimes contains a single speck of cartilage) but none of them had distinct polydactylous digits (18/18 hindlimbs; Fig 2.10O). *Gremlin*$^{\Delta GRE1/\Delta GRE1}$ littermates have normal digit patterning (14/14 hindlimbs; Fig 2.10N). In contrast, *Gremlin*$^{\Delta GRE1/\Delta GRE1}$;*Gli3*$^{+/-}$littermates have a distinct, polydactylous digit in 3/8 hindlimbs (Fig 2.10P), a significant difference from

Gli3$^{+/-}$ embryos alone (p =0.0215). *Gli3*$^{+/-}$ forelimbs displayed a spectrum of phenotypes ranging from completely normal digits (7/17) to polysyndactyly (4/17). *Gremlin*$^{\Delta GRE1/\Delta GRE1}$;*Gli3*$^{+/-}$ forelimbs uniformly contained a polysyndactylous thumb (8/8), a significant increase in frequency compared to *Gli3*$^{+/-}$ embryos (p = 0.0005; Fig 2.10I-L). *Gremlin*$^{\Delta GRE1}$;*Gli3*$^{+/-}$ embryos also contained a high proportion of polysyndactylous forelimbs (23/28). These results suggest that GRE1 has silencer activity that is required for robust anterior repression of *Gremlin*. Our result is consistent with previous studies showing a genetic interaction between GLI3 and BMP4 (Dunn et al., 1997; Lopez-Rios et al., 2012). To determine if GRE1 might also be required to provide a GLI activator input for robust GLI enhancer activity, we performed a parallel analysis of compound crosses with *Shh*$^{-/-}$ mice. *Gremlin*$^{\Delta GRE1/\Delta GRE1}$;*Shh*$^{+/-}$ embryos have no genetic interaction, suggesting that the enhancer properties of GRE1 are either completely redundant or biologically irrelevant. We concluded that silencer activity through GRE1 is required for robust, GLI-dependent repression of *Gremlin* in the anterior limb (schematized in Fig 2.10Q-T).

2.7 DISCUSSION

In this study we have performed the first genetic characterization of a vertebrate GLI CRM. Within the limb bud, most putative GLI target genes are associated with multiple GLI binding regions (Vokes et al., 2008). Our results suggest that one role for multiple, distinct GLI binding regions around GLI target genes is to provide a robust silencing response that buffers against genetic perturbations. This contrasts with the *Fgf8* and *HoxD* loci where multiple enhancers with similar activity domains have been proposed to additively or synergistically amplify transcription (Marinic et al., 2013; Montavon et al., 2011). Our results further suggest that GLI silencers prevent

transcriptional activity driven by additional, GLI-independent CRMs. We also show that GRE1 also has GLI-activator dependent enhancer activity in the posterior limb. Thus, the GRE1 CRM provides a platform for GLI activator and GLI repressor to respectively enhance or silence *Gremlin* expression. We propose a model where GLI repressors bind to multiple GLI-dependent CRMs in the anterior limb, providing a robust silencing activity that prevents ectopic activation of *Gremlin* that would otherwise be driven by at least one additional GLI-independent CRM that is active throughout the distal limb (a pan-limb enhancer). GLI repressor-mediated silencing results in the anterior repression of *Gremlin* in the absence of threshold levels of GLI activator complexes. In the posterior limb, where GLI activator activity is high and GLI repressor activity is low, GRE1 silencing activity is lost and GLI-activator complexes provide enhancer activity. We have synthesized these results in a model for how *Gremlin* is regulated by GLI proteins within the limb (Fig 2.11).

2.7.1 GLI ENHANCER ACTIVITY

GRE1 enhancer activity is detected in the posterior limb in a spatial and temporal fashion that correlates with Shh signaling (Fig 2.1). GRE1 requires GLI-activation for initiating and sustaining activity at E10.5 and ectopic GLI activator signaling is sufficient to drive GRE1 expression throughout the anterior-posterior axis (Fig 2.2). These results suggest that GRE1 enhancer activity is primarily regulated by Shh signaling. GRE1 enhancer activity is transiently reduced in E10.5 *Gli3*$^{-/-}$ limb buds (Fig 2.2D). Our own results (Fig 2.3) are consistent with several studies showing that *Gli3*$^{-/-}$ limbs have reduced levels of GLI activation caused by a combination of reduced levels of GLI proteins and a reduction in *Shh* (Bai et al., 2004; Bowers et al., 2012; Galli et al., 2010;

Wang et al., 2007).

In marked contrast to *Gremlin* gene expression, the GRE1 enhancer domain does not expand in E10.5 *Gli3*$^{-/-}$ limb buds (Fig 2.2D,D''). We rule out the trivial possibility that GLI repressors do not work through GRE1 because our subsequent experiments indicate that it does indeed mediate GLI repressor-mediated silencing of *Gremlin* (Figs 2.7, 2.10) and it is bound by GLI3 repressor in chromatin immunoprecipitation assays (Vokes et al., 2008). The behavior of GRE1 contrasts with the behavior of a *Dpp* wing imaginal disc CRM in *Drosophila*, where both repressor and activator functions of Ci can be detected out in the same enhancer element (Muller and Basler, 2000). Within the mammalian neural tube, studies have reported conflicting conclusions regarding the role for GLI3 in restricting the boundaries of GLI activator enhancers or genes (Balaskas et al., 2012; Oosterveen et al., 2012; Peterson et al., 2012).

In contrast to the absence of anterior expression at E10.5, there is a thin, anterior domain of enhancer activity at E11 in *Gli3*$^{-/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos. Previous experiments with G0 transgenics indicated that a GLI motif within GRE1 is absolutely required for enhancer activity at E11 (Vokes et al., 2008). It is presently unclear whether this expression reflects a weak, late role for GLI3 repression in restricting the enhancer domain, an artifact of the enhancer construct or transgenic line or some type of indirect activation. If this represents biological derepression, a possible model would be the presence of an unknown anterior activator in the anterior limb that is repressed by GLI3 but activates late GRE1 activity. This would be consistent with our previous G0 transgenic enhancer results because they were in wild-type limbs and so the hypothetical anterior activator would still be repressed (Vokes et al., 2008).

There are several possible explanations for the lack of anterior expansion of *GRE1LacZ* in E10.5 *Gli3*$^{-/-}$ limb buds. The first is that GLI repressors might not compete

with GLI activators to limit the anterior domain of enhancer activity. In this scenario, enhancer activity is driven solely by threshold-dependent GLI activation. The lack of baseline anterior activity would prevent visualization of the silencer activity in an enhancer reporter assay. A second possibility is that the *GRE1LacZ* transgenic construct is incapable of responding normally to GLI repressors because it is removed from its normal chromosomal environment. Indeed, our experiments suggest that GLI repressors do regulate the activity of additional CRMs in the *Gremlin* locus (Figs 2.10, 2.11). Taken out of context, GRE1 could also have altered affinities for GLI activator and repressor complexes that prevent its anterior expansion in *Gli3*$^{-/-}$ embryos. A third possibility is that residual GLI repressor activity is sufficient to prevent anterior expansion of *GRE1LacZ* in *Gli3*$^{-/-}$ limb buds. Consistent with this, recent work has indicated that there is a genetic role for GLI2 repressor in skeletal patterning in the absence of GLI3 (Bowers et al., 2012). However, *Gremlin* expression appears largely symmetrical along the anterior-posterior axis in *Gli3*$^{-/-}$ limb buds, suggesting that the remaining GLI repressor activity mediated by GLI2 might not be sufficient to repress *Gremlin* (Fig 2.2D') (Aoto et al., 2002; Litingtung et al., 2002; te Welscher et al., 2002). Additional studies examining *GRE1LacZ* enhancer activity in *Gli2*$^{-/-}$*; Gli3*$^{-/-}$ limb buds would be necessary to determine if GRE1 itself is more sensitive to GLI2 repression than the overall *Gremlin* gene expression pattern would suggest.

2.7.2 GLI REPRESSOR ACTIVITY

Two models for GLI repression have been proposed. In one, GLI3 repressor acts as an inert decoy competing with GLI activator to regulate the transcription of target genes. In the second, GLI repressor behaves like a conventional transcriptional repressor,

recruiting transcriptional co-repressors that actively shut down transcription (Oosterveen et al., 2012; Wang et al., 2010). While the first model would apply specifically to GLI activator target genes, the second model could in principle apply to both GLI activator target genes and to genes that only require GLI derepression. GRE1 displays properties that are associated with both classes of GLI target genes. *Gremlin* is a GLI derepression gene and GLI3 works through GRE1 as a silencer, preventing transcription directed by additional CRMs that would otherwise lead to ectopic distal-anterior expression. In future studies, it will be interesting to determine mechanism of repression, which could function as a basal regulator of transcriptional activity. Alternatively GLI3 might specifically inactivate one or more CRMs.

2.7.3 GLI PROTEINS GENERATE ASYMMETRIC GENE EXPRESSION

In the posterior limb bud, it is unclear whether GLI activators are simply indicative of a de-repressed environment that permits additional CRMs to drive expression or if they also provide a quantitative contribution as enhancers to increase *Gremlin* transcription. The only evidence suggesting GRE1 is an enhancer is the enhancer activity of the isolated element in transgenic limb buds. While this fits the generally accepted criteria for an enhancer, there is no genetic evidence for reduced GLI activator responses in either $Gremlin^{\Delta GRE1/+}$ or $Gremlin^{\Delta GRE1/\Delta GRE1}$;$Shh^{+/-}$ embryos(Fig 2.12). There is also no observable reduction of posterior β-galactosidase activity in the BAC transgenics harboring a deletion in GRE1 (Fig 2.7B). The lack of any detectable phenotype suggests that in the context of the native genomic locus, the enhancer activity is absent, trivial or completely redundant with additional GLI-dependent CRMs. The ambiguity over the contribution of enhancer activity is represented in Fig 2.11,

suggesting that the major purpose of GRE1 enhancer activity lies in counteracting GLI repression rather than providing quantitative levels of activation. In this way, GRE1 could act as a binary switch, causing transcription to be on or off in different domains. This model provides a mechanism for how Shh signaling imposes asymmetric expression of 'pre-patterned' genes that would, in the absence of any GLI regulation, be symmetrically expressed throughout the limb bud. It also suggests that the inclusion of GLI-driven CRMs into the locus of pre-patterned limb might have provided an evolutionary mechanism for regulating asymmetric gene expression in a pre-existing pattern.

2.7.4 MULTIPLE CRMS REGULATE GREMLIN

Within the context of this study, there appear to be at least three distinct CRMs regulating *Gremlin*. This is consistent with previous studies that describe a complex regulatory locus for *Gremlin* (Vokes et al., 2008; Wang et al., 1997; Zuniga et al., 2012; Zuniga et al., 2004). Several proteins have also been shown to regulate *Gremlin* at various developmental timepoints. In particular, BMPs and HoxA/D transcription factors both regulate *Gremlin* along the anterior-posterior axis. Their activity and expression domains make them excellent candidate regulators for the GLI-independent pan-limb enhancer (Fig 2.11) (Benazet et al., 2009; Capdevila et al., 1999; Nissim et al., 2006; Sheth et al., 2013). Intriguingly, HoxA/D conditional mutants lack most *Gremlin* expression with the exception of a posterior domain that appears nearly identical to the GLI CRM enhancer domain (Fig 2.1D) (Sheth et al., 2013). Although our model depicts pan -limb enhancer activity with one CRM as the simplest possibility (Fig 2.11), it is

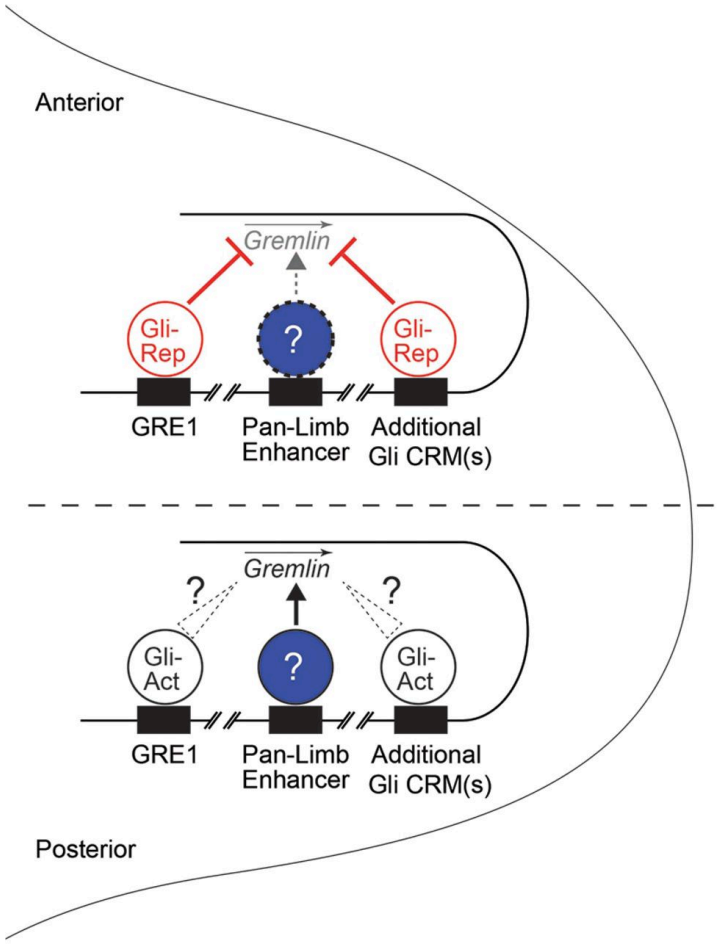

Figure 2.11 GLI proteins generate asymmetric expression of *Gremlin*

Gremlin is activated by a pan-limb enhancer (blue circle) that has activity throughout the distal limb. In genetic backgrounds in which there is an absence of GLI regulation (no activation or repression, e.g. *Shh*$^{-/-}$;*Gli3*$^{-/-}$), the pan-limb enhancer drives symmetrical expression of *Gremlin* throughout the limb bud. In the posterior region, GLI activators regulate redundant GLI-dependent CRMs, including GRE1, causing a loss of GLI-mediated silencing and possibly threshold-dependent enhancer activity (indicated by dashed triangles). In the anterior region, GRE1 acts as a silencer, preventing ectopic activation of *Gremlin* through a pan-limb enhancer. The additional GLI-dependent CRM(s) could be either directly or indirectly regulated by Hedgehog signaling.

certainly possible that this activity integrates multiple GLI-independent enhancers active in distinct or overlapping domains.

Recently, a second *Gremlin* CRM that lies closer to the transcriptional start site has been characterized. While it does not contain a high affinity GLI motif within the core region, it is nonetheless bound by GLI3 in ChIP assays and requires Shh expression for enhancer activity in mutant embryos (Zuniga et al., 2012). Unlike GRE1, the more proximal CRM is essential for *Gremlin* transcriptional activity in the same BAC reporter used in this study (Zuniga et al., 2012). Notably, GRE1 is not sufficient to activate transcriptional activity in its absence. This more proximal CRM could integrate GLI signaling with additional, Shh-independent, facets of Gremlin or there could be additional, uncharacterized GLI-dependent element(s). Our study was limited to the contribution of a single CRM, and future studies will be required to determine if there are higher-order chromatin interactions among the individual CRMs regulating Gremlin as has been suggested for the Fgf8 and HoxD loci (Marinic et al., 2013; Montavon et al., 2011). In *Drosophila*, Ci repressors have been proposed to work cooperatively by binding to several distinct sites within a CRM regulating Dpp (Parker et al., 2011). The presence of an additional GLI CRM in the Gremlin locus raises the intriguing possibility that GLI proteins binding to distinct CRMs might nonetheless be able to cooperatively repress Gremlin in the context of a higher order chromatin structure.

2.7.5 REDUNDANT GLI INPUT AS A MECHANISM FOR FOSTERING ROBUST TRANSCRIPTIONAL CONTROL

Given the critical role for Shh in regulating *Gremlin* and the significant de-repression observed when GRE1 was deleted in transgenic BAC reporters (Fig 2.4B), we

were initially surprised at the subtle phenotypes seen upon deleting the GLI CRM. Embryos and mice lacking GRE1 have no detectable skeletal phenotype. Nonetheless, embryos do have subtle shifts in *Gremlin* expression (Fig 2.6A,B), and when one copy of *Gli3* is removed GRE1 is required for the repression of *Gremlin*. It is formally possible that the enhanced phenotype seen in *GRE1*;*Gli3* compound heterozygous embryos (Fig 2.6) is due to the presence of another allele co-segregating with GRE1. The primary support that this interaction occurs between GRE1 and *Gli3* is that it is consistent with interactions observed between GLI3 and BMPs (which should have reduced anterior activity with ectopic *Gremlin* expression) (Dunn et al., 1997; Lopez-Rios et al., 2012). Both the subtle changes in expression pattern and the requirement of the CRM as a mechanism for buffering genetic variation are analogous to the shadow enhancers described in *Drosophila* (Barolo, 2012; Frankel et al., 2010). Shadow elements are defined by the genetic interactions of two genetically defined CRMs (Frankel, 2012) and further genetic studies involving multiple GLI bound elements would be required to determine if the GLI CRM is functioning as a shadow repressor of *Gremlin*. Our study, focused exclusively on a single GLI CRM, is the first to address the potential genetic role that multiple GLI-bound CRMs play in regulating transcription. Multiple GLI binding sites are associated with many predicted GLI target genes (Peterson et al., 2012; Vokes et al., 2008) and we propose that they may act as a general mechanism for mediating robust transcriptional responses to Hedgehog signaling.

Gremlin is an important Shh target gene in limb buds. Its expression is absent in $Shh^{-/-}$ limb buds but rescued in $Shh^{-/-};Gli3^{-/-}$. This suggests *Gremlin* is a Shh de-repression gene whose expression is driven by a Shh-independent enhancer, but suppressed by GLI3-mediated transcriptional repression. We previously identified three GLI3 ChIP enrichment peaks (Peak1-3) in the *Gremlin* regulatory locus. GRE1 (Peak1), one of the

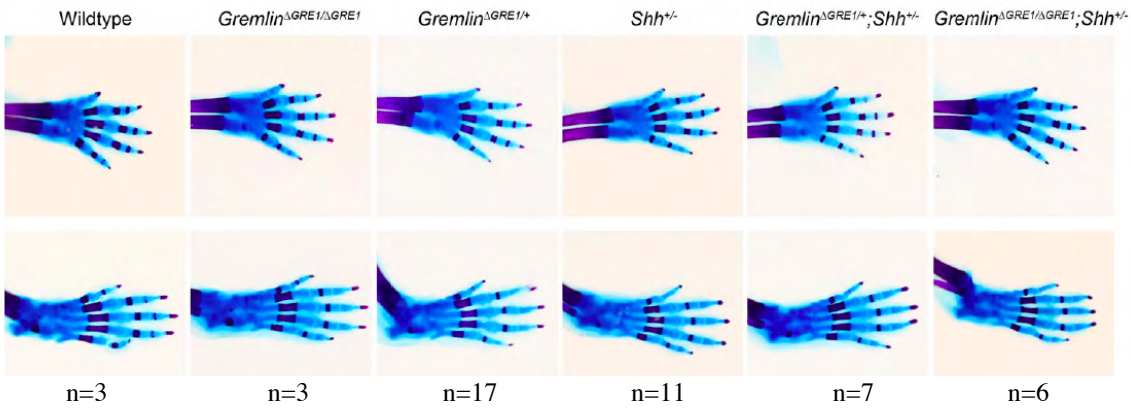

Figure 2.12 The *Gremlin*$^{\Delta GRE1}$ allele does not genetically interact with *Shh*

Gremlin$^{\Delta GRE1/+}$;*Shh*$^{+/-}$ males were crossed to *Gremlin*$^{\Delta GRE1/+}$ females and their litters were analyzed by skeletal preparations at E18.5 stained for bone (Alizarin Red) and cartilage (Alcian Blue). Representative hands from forelimbs are shown on the top row. The second row shows representative feet. The numbers of skeletons that were analyzed for each genotype are indicated below. All skeletal preparations were normal with the exception of a small, fleshy nub observed on one hindlimb of *Gremlin*$^{\Delta GRE1/\Delta GRE}$ skeleton (something we have never otherwise observed).

three peaks has been verified as a Shh-dependent CRM that acts as either an enhancer or repressor in a context-dependent fashion (Li et al. 2014b). I further test the potential roles of the other two GLI3 enriched regions in *Gremlin* regulation by G0 transient transgenic enhancer assays.

To gain better understanding of the other two GLI enriched regions in the *Gremlin* regulatory locus (Zuniga et al. 2004), I cloned the two regions (indicated as "Peak2", and "Peak3", Fig 2.13) into a minimal-promoter *LacZ* reporter construct and tested in G0 transient transgenic for enhancer activity. Peak2 did not exhibit any enhancer activity in the embryonic limb buds (Fig 2.13C), however, Peak3 exhibited a strong pan-limb enhancer activity (Fig 2.13D). As shown in Fig 2.13D, Peak3 drove *LacZ* reporter

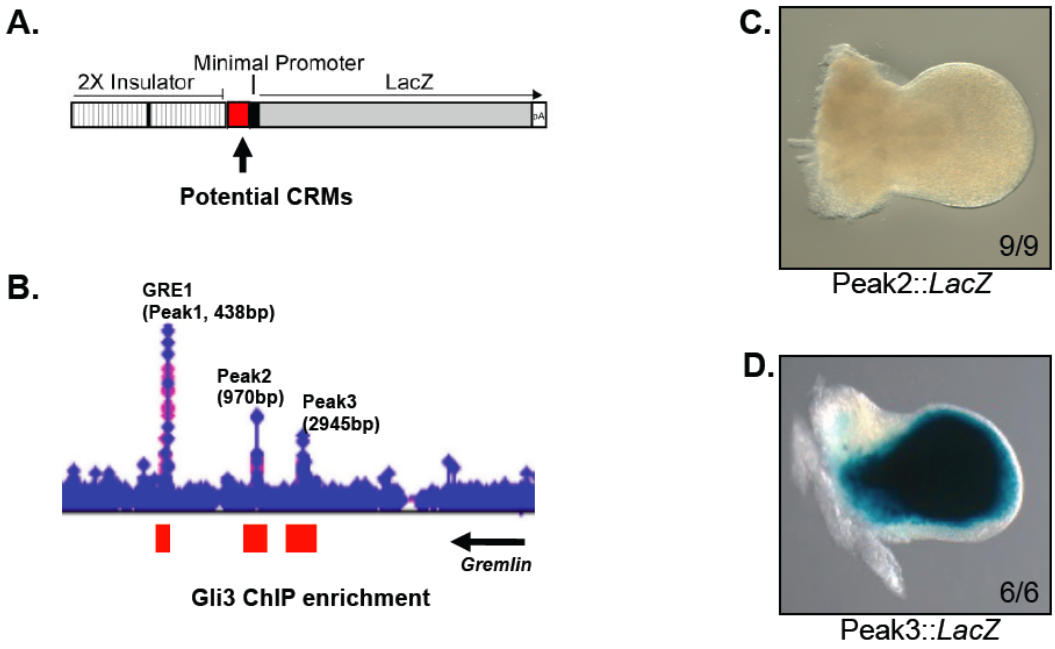

Figure 2.13 Enhancer activities of GLI3 enriched regions located in *Gremlin* regulatory locus

A: Schematic of minimal-promoter *lacZ* reporter. B: Schematic of the three GLI3 ChIP enrichment peaks. C: Peak2 has no enhancer activity in embryonic limb buds. D: Peak3 acts as a pan-limb enhancer in our G0 transient transgenic test. Peak3, but not Peak2, shows strong enhancer activity throughout the entire limb bud in G0 transgenic assay. Peak3::*LacZ* expression domain is much boarder than the Shh responsive domain, suggesting Peak3 might be a Shh-independent limb enhancer.

gene expressing throughout the entire limb and the expression pattern extended into the proximal region where *Gremlin* gene expression normally does not existed.

In order to test the silencer activity of GLI3 ChIP peaks, the 906bp human limb specific enhancer hs72(VISTA Enhancer Browser) was cloned into the minimal promoter *LacZ* reporter (hs72::*LacZ*) as control, and compared to reporters with Peak1 or Peak2 sequence downstream of hs72, respectively (hs72_Peak1::*LacZ* and hs72_Peak2::*LacZ*). As predicted, when tested in G0 transgenics, hs72::*LacZ* alone drives *LacZ* expressed

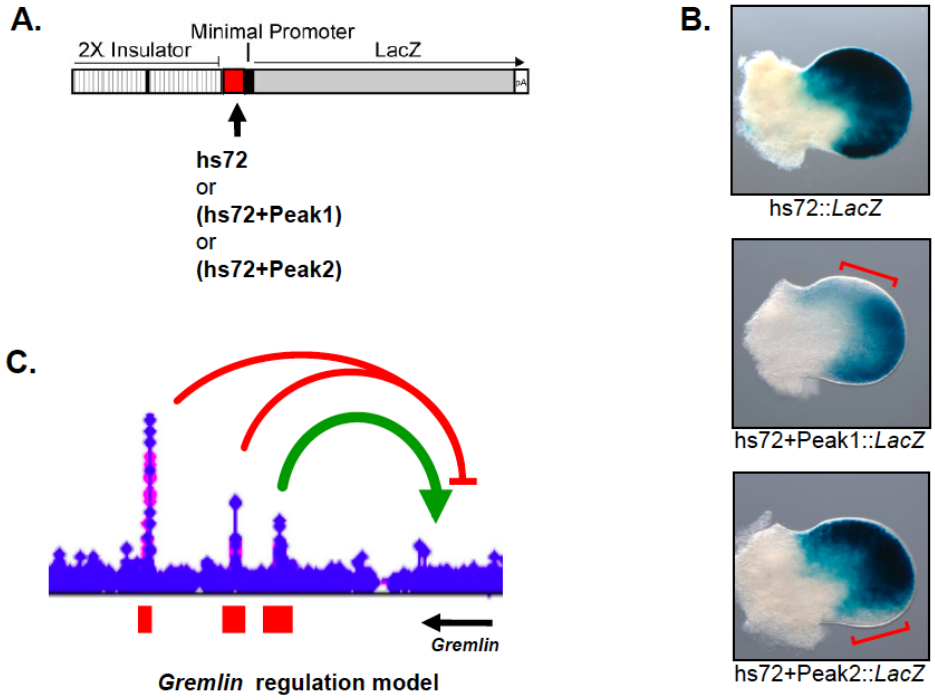

Figure 2.14 The *Gremlin* regulation model

A: Schematic of testing silencer activity of Peak1 (GRE1) and Peak2. B: hs72 is a limb specific enhancer, and its expression pattern was limited at either anterior or posterior region by adding Peak1 or Peak2 respectively. C: Schematic of the "*Gremlin* regulation model". In this model, Peak3 acts as a "Pan-limb enhancer", and Peak1(GRE1) and Peak2 provide fine tuning of transcriptional regulation and maintain the normal *Gremlin* asymmetric expression pattern.

throughout the entire limb bud, while Peak1 impaired *LacZ* expression at anterior and Peak2 repressed its expression in the posterior-distal region (Fig 2.14).

Our genetic study of GRE1 (Peak1) suggests that *Gremlin* is likely to be repressed by multiple redundant CRMs; the observation from our lab that *Gremlin* expression is fully rescued in $Shh^{-/-};Gli3^{-/-}$ also suggests that *Gremlin* needs Shh signals for de-repression but not activation. Thus, I propose a "*Gremlin* regulation model": during

limb development, *Gremlin* is driven by Peak3, an Shh-independent enhancer in the entire limb bud, but repressed by GLI3 through Peak1 and Peak2, two redundant cis-regulatory modules that maintain the normal expression pattern.

Chapter 3: NANOG binds to GLI proteins in ES cells and represses Hedgehog signaling mediated transcription

The Hedgehog pathway was initially characterized for regulating embryonic development but it also has critical roles in regulating the homeostasis of several adult tissues (reviewed in Petrova and Joyner, 2014). One of the best-characterized roles of Shh lies in its roles in regulating two major neural stem cell populations in the brain: the ventral sub-ventricular zone (VSVZ) and sub-germinal zone (SVZ). In the absence or inhibition of the Hedgehog pathway, both the VSVZ and the SVZ undergo a marked depletion in cells, indicating that the pathway is required for normal proliferation (reviewed in Alvarez-Buylla and Ihrie, 2014). Conversely, hyperactivation of the Hedgehog pathway results in an expanded population of neural stem cells. In this context, the progeny of neural stem cells is shifted so that they preferentially give rise to two daughter stem cells instead of producing transit amplifying cells capable of generating differentiated progeny (Ferent et al., 2014). Together these results indicate that the levels of Hedgehog perceived by neural stem cells regulate the balance between generating stem cells and differentiated progenitors.

In addition to regulating normal neural development, various studies have suggested populations of stem cells play key roles in medulloblastoma as well as glioblastoma (Po et al., 2010, Vanner et al., 2014). In glioblastoma cancer models, GLI proteins have been shown to activate the transcription of the pluripotency factor NANOG. NANOG in turn is critical for maintaining tumorigenic cell populations, suggesting a positive feedback between these factors (Po et al., 2010; Zbinden et al., 2010).

While Hedgehog signaling via GLI transcription factors is critical for regulating

neural stem cells, the underlying transcriptional mechanisms remain poorly understood. In part, this is because it is difficult to isolate large numbers of these stem cells. In an effort to understand this process, we performed a mass-spectroscopy based screen to identify GLI binding proteins in mouse embryonic stem cells that might act as stem cell-specific co-factors. Here, we report that GLI1 and GLI3 bind to the pluripotency factor NANOG. We find that the presence of NANOG inhibits GLI transcriptional responses in several different cell types. Interestingly, interactions between NANOG and GLI proteins have previously been implicated in regulating brain tumors (Po et al., 2010; Zbinden et al., 2010). Finally, we show that NANOG acts to dampen the response of ES cells to Hedgehog signaling. Previous studies have shown that NANOG also binds to and inhibits the BMP and NFκB pathways (Suzuki et al. 2006; Torres and Watt, 2008) in ES cells thus NANOG may play a more general role in insulating ES cells from the surrounding signaling environment.

3.1 NANOG BINDS TO GLI3 IN ES CELLS

To identify GLI associated proteins in stem cells, we utilized mouse embryonic stem (ES) cells. When cultured under the appropriate conditions, these cells remain pluripotent, allowing us to obtain large amounts of material. The ES cells contained a tamoxifen-inducible 3XFLAG-tagged Gli3 repressor (GLI3R) allele driven by the ubiquitous Rosa26 promoter, an allele that has previously been used to generate mice for chromatin immunoprecipitation experiments (ChIP) (Vokes et al., 2008). We performed anti-FLAG immunoprecipitation (IP) followed by mass spectrometry on lysates from tamoxifen-induced ES cells (expressing Flag-tagged GLI3R) and parental, control ES cells (not expressing Flag-tagged GLI3R). We sorted the resulting list based on the Z-

Protein Name	Accession #	Z Scores	Spectra counts#1	Spectra counts#2	Spectra counts#3
GLI3	P61602	15.34	60	90	104
Aldolase 1	P05064	3.11	4	12	0
Desmoplakin*	E9Q557	3.04	3	13	0
TCP-1 beta	P80314	1.97	3	3	0
DAX1	Q61066	1.79	3	2	0
Sm-D2	P62317	1.78	0	0	11
Prelamin-A/C	P48678	1.62	0	8	0
ADE2	Q9DCL9	1.61	2	2	0
NANOG	Q80Z64	1.61	2	2	0
MYL6	Q60605	1.61	0	0	9
Sm-D1	P62315	1.61	0	0	9
SON	Q9QX47	1.61	0	0	9
TER ATPase	Q01853	1.60	8	0	0
SUFU	Q9Z0P7	1.57	0	2	2
Acin1	Q9JIX8	1.51	0	7	0
14-3-3	Q70456	1.51	0	7	0

Table 3.1 GLI3R-interacting proteins identified by mass spectrometry

Proteins identified in GLI3R protein complexes by mass spectrometry are sorted based on Z score with a cutoff value of 1.5. Gray shade highlights the bait protein and two known GLI-interacting proteins. Green shade highlights the two pluripotency factors. *ES cell lysate background proteins (Wang et al. 2006)

score enrichment (Lu et al. 2007) with a cutoff value of 1.5, resulting in 27 proteins. We then removed proteins that contained any spectral counts in control cells, resulting in a final list of 16 proteins (Table 3.1). The most enriched protein in this list was the bait protein, GLI3. This list contained two proteins, SUFU and 14-3-3, which have previously been shown to bind GLI proteins (Humke et al., 2010; Asaoka et al., 2010). The remaining 13 proteins have not previously been associated with GLI proteins. Interestingly NANOG, a well established pluripotency factor and its co-factor DAX-1 (Wang et al., 2006)) were both present on this list and we decided to focus our subsequent efforts on characterizing this interaction.

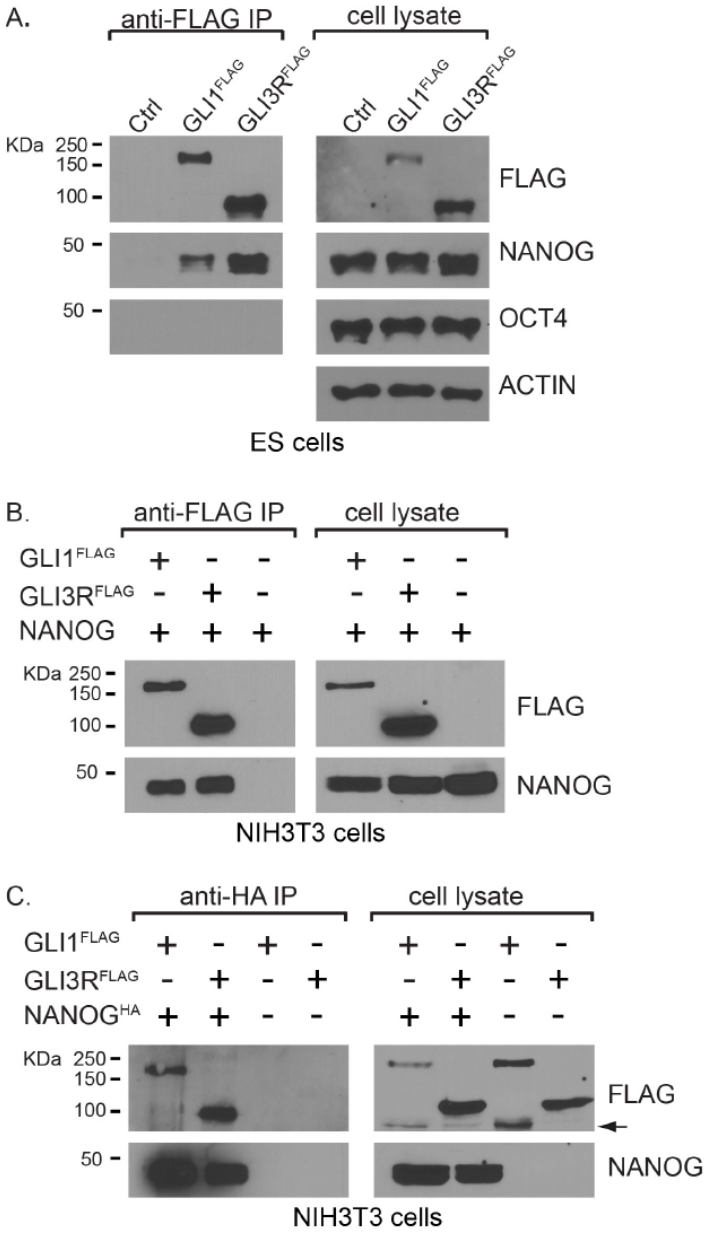

Figure 3.1 NANOG interacts with both GLI activator and repressor

A: Endogenous NANOG, but not OCT4, co-immunoprecipitated with both FLAG-tagged GLI activator ($GLI1^{FLAG}$) and GLI repressor ($GLI3R^{FLAG}$) in an anti-FLAG IP with $GLI1^{FLAG}$ and $GLI3R^{FLAG}$ expressing ES cell lines. B, C: Interactions between overexpressed GLI proteins and NANOG in NIH3T3 cells were verified by either anti-FLAG immunoprecipitations (B) or anti-HA immunoprecipitation (C). Co-transfected GLI1/GLI3R and NANOG plasmids used in each experiment are indicated above the blots. The arrow labels a non-specific band.

To validate the interaction between GLI3R and NANOG, we performed additional immunoprecipitations(IP) by western blots (Fig 3.1A). NANOG is a core regulator of stem cells and acts in conjunction with SOX2 and OCT4 to maintain ES cell self-renewal and pluripotency (reviewed in Young 2011). However, OCT4 was not co-immunoprecipitated with NANOG with GLI3R (Fig 3.1A). This suggests that the NANOG interacts with GLI3R in a complex that is distinct from NANOG::OCT4.

3.2 NANOG INTERACTS WITH BOTH GLI ACTIVATORS AND GLI REPRESSORS

The previous experiments demonstrated that NANOG binds to GLI3R. To determine if NANOG might also be able to interact with additional GLI proteins, we used an ES cell line containing a Cre-inducible, 3XFLAG-tagged full length Gli1 to perform additional immunoprecipitations. Unlike GLI2 and GLI3, GLI1 exists only as a full-length protein where it acts as a transcriptional activator (reviewed in Hui and Angers, 2011). As shown in Fig 3.1A, GLI1 binds to NANOG but not OCT4.

To confirm that NANOG interacts with both GLI1 and GLI3R, we co-transfected NIH3T3 cells with constructs encoding HA-tagged NANOG and either 3XFLAG-tagged GLI1 or 3XFLAG-tagged GLI3R. Consistent with the previous interactions in ES cells, NANOG binds to both GLI1 and GLI3R (Fig 3.1B, C)

3.3 NANONG REPRESSES GLI1-MEDIATED TRANSCRIPTIONAL ACTIVATION

NANOG helps to maintain ES cell self-renewal and pluripotency in part by repressing key differential regulatory genes (reviewed in Young, 2011) To determine if NANOG influences GLI-mediated transcription, we first utilized a GLI responsive luciferase assay in NIH3T3 cells. Compared to cells transfected with GLI1 alone, cells

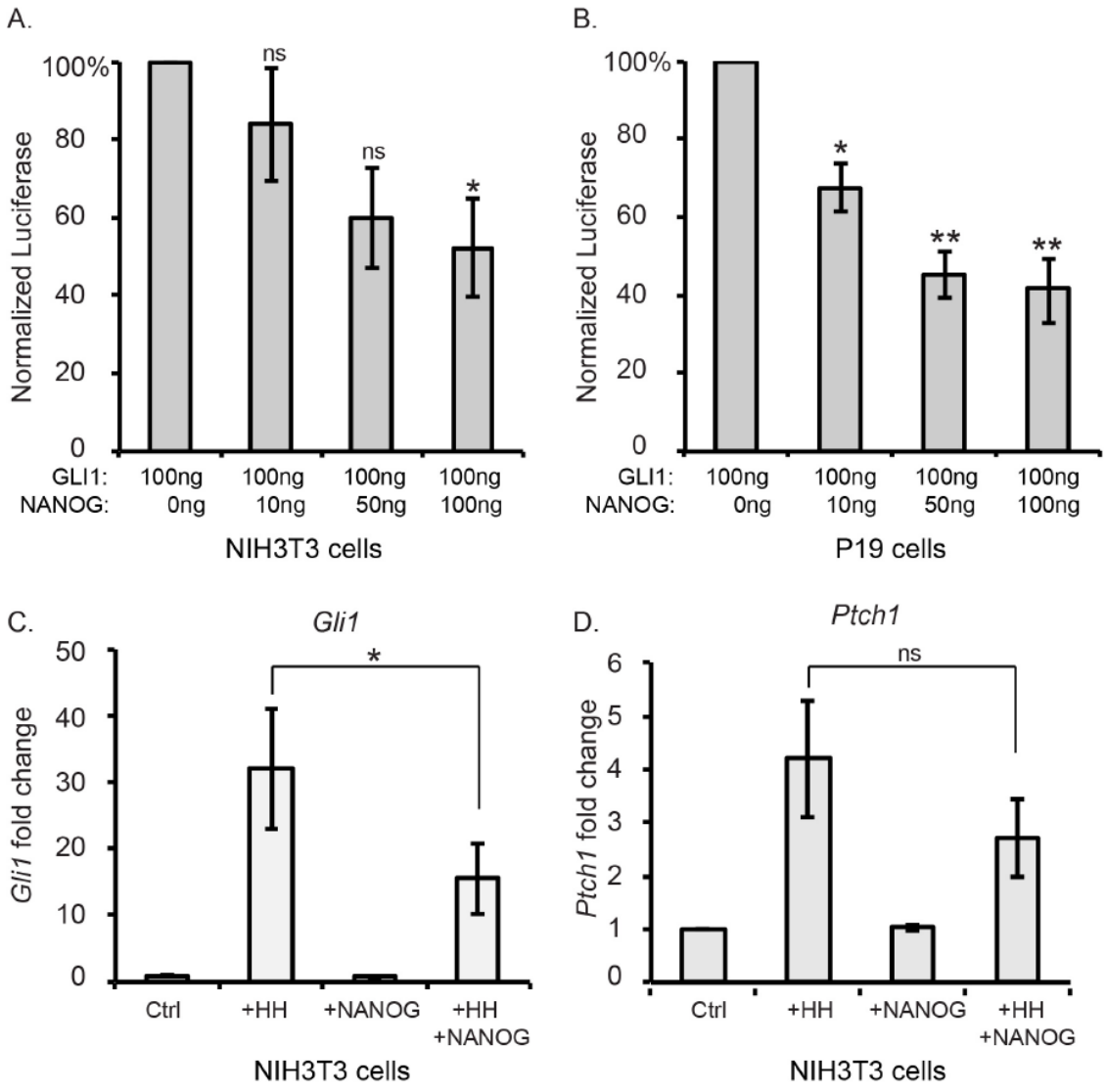

Figure 3.2 NANOG represses GLIA-mediated transcription activation

A, B: GLI1-mediated Ptch-Luciferase activity was repressed by co-transfecting with indicated amounts of a NANOG expressing vector, in both NIH3T3 cells and P19 cells. Hedgehog signaling responsive Ptch-Luciferase reporter was co-transfected with indicated amount of GLI1 and NANOG expressing plasmids into NIH3T3 cells and luciferasae assay was performed as in described in Materials and Methods. C: RT Q-PCR of Hedgehog target genes *Gli1* and *Ptch1* in NIH3T3 cells upon purmorphamine (+HH) stimulation, in the absence and presence of ectopically expressing NANOG. Statistical significances were measured by a two-tailed, paired T-test from three independent experiments, *:$P<0.05$, ns: not significant, $0.1>P>0.05$.

transfected with both GLI1 and NANOG had a dose dependent reduction in luciferase activity (Fig 3.2A). We noted a similar trend in the embryonic carcinoma P19 cells, where co-expression of NANOG reduced GLI1-mediated luciferase response (Fig 3.2B). To determine if NANOG expression also inhibited the expression of endogenous Hedgehog target genes, we treated NIH3T3 cells with or without purmorphamine, a small molecule that activates Hedgehog signaling (Sinha and Chen, 2006) and performed quantitative real time PCR (q-PCR) on cDNA. As expected, NIH3T3 cells treated with purmorphamine had increased expression of the Hedgehog target genes *Ptch1* and *Gli1* compared to untreated cells (Fig 2C, D). When cells were transfected with NANOG, there is a significant 53% reduction in *Gli1* ($p=0.0482$). Although not statistically significant ($p=0.0524$), there is a 35% reduction in *Ptch1*, suggesting a similar trend.

3.4 NANOG BINDS TO GLI1 THROUGH ITS C-TERMINAL DOMAINS

NANOG is a 305 amino acid protein contains a conserved homeodomain (95-155aa) at the N-terminus and a tryptophan repeat (WR) domain (197-244aa) at the C-terminus (Chambers et al., 2003; Mitsui et al., 2003). To identify the protein binding domain on NANOG, we generated a series of HA-tagged NANOG truncations (Fig 3.3A) and co-transfected them with FLAG-tagged-GLI1 (Fig 3.3B). As expected, full length NANOG was co-immunoprecipitated with GLI1. Constructs lacking the C-terminal half of NANOG did not interact with GLI1, indicating that the C-terminal half is essential for the interaction. To determine if the C-terminal region could bind NANOG, we generated additional constructs C1, C2 and C3 but were unable to obtain comparable expression levels to full-length NANOG and N1 most likely because of their small size (data not shown). To circumvent this, we generated additional fusion proteins containing a larger,

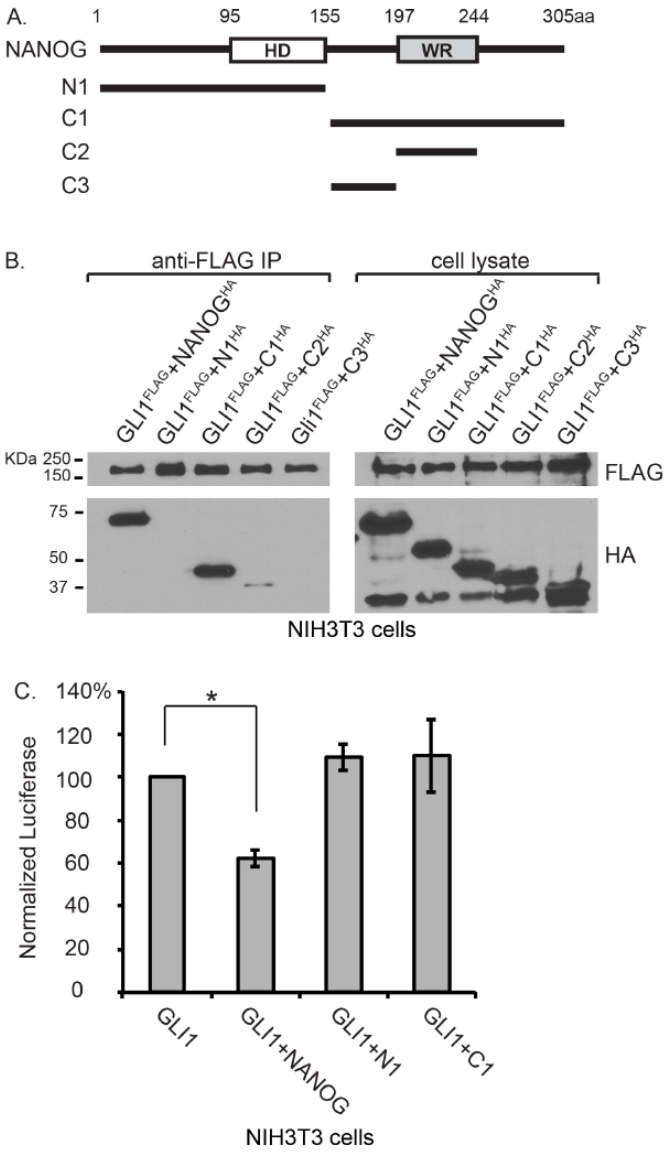

Figure 3.3 Function analysis of different NANOG domains

(A).Schematic diagram of full-length NANOG and truncations that were tested in this study. HD: Homeodomain, WR: Tryptophan Repeat domain. (B),(C).Anti-FLAG immunoprecipitation with cell lysates from NIH3T3 cells co-transfected with FLAG-tagged GLI1 and HA-tagged NANOG or truncations expressing vectors. (D).GLI1-mediated Ptch::Luciferase activity was assayed in the presence of indicated Nanog truncations. Statistical significances were measured by a two-tailed, paired T-test from three independent experiments, *:P<0.05

HA-YFP tag that resulted in comparable levels of expressions (Fig 3.3C). The C1 truncation bound GLI1 at levels that were comparable to full-length NANOG (Fig 3.3C), suggesting that the C-terminal region of the NANOG is responsible for binding GLI1. We generated additional subdomains of the C-terminal half (C2, C3). The C2 construct bound only minimally to GLI1 the while C3 did not bind at all. CD2 itself was not capable to bind GLI1. These results suggest that an extensive region of the C-terminal region of NANOG binds to GLI1. Interestingly, this C-terminal half of NANOG is also critical for interaction between NANONG and the NFκB family transcription factors (Torres and Watt, 2008), which suggests NANOG may contain a general motif that interacts with distinct transcription factors.

3.5 THE N-TERMINUS OF NANOG IS ESSENTIAL FOR REPRESSING GLI1-MEDIATED TRANSCRIPTION

The previous results indicated that the C-terminal region of NANOG (construct C1) is sufficient to bind GLI1. We next asked whether this region was also sufficient to inhibit GLI1-mediated transcription. To test this, we co-transfected GLI1 with specific NANOG truncation constructs and a GLI-responsive luciferase construct. Although C1 robustly binds to GLI1, it does not repress GLI1-mediated transcription. As expected, GLI response was not reduced when the N-terminal region of NANOG (construct N1) was co-transfected with GLI1 (Fig 3.3D). The simplest interpretation of these results is that the N-terminus of NANOG inhibits GLI repression. This is consistent with other studies showing that the N-terminal portion of NANOG contains a transcriptional repressor motif (Chang et al., 2009)

3.6 HEDGEHOG SIGNALING UPREGULATES NANOG PROTEIN LEVELS IN DIFFERENTIATING ES CELLS

In an effort to examine the significance of the NANOG-GLI interactions in ES cells, we activated Hedgehog signaling with the small molecule purmorphamine either under conditions that main stem cell growth or cause differentiation (by the withdrawal of Leukemia inhibitory factor, "-LIF"). In the presence of LIF, the expression of NANOG was highly expressed and unaffected by the stimulation of Hedgehog signaling (Fig 3.4A). As expected, ES cells began differentiating upon LIF withdrawal and after 4 days expressed markedly lower levels of NANOG Interestingly, upon Hedgehog stimulation, NANOG levels were upregulated upon LIF withdrawal (Fig 3.4A)

We next compared the ability of GLI to form complexes with NANOG in the presence and absence of Hedgehog signaling. Although NANOG was not upregulated by Hedgehog stimulation under normal conditions that maintained proliferation (Fig 3.4A), there was a marked increase in the amount of NANOG pulled down by GLI1 (Fig 3.4B). Because the GLI1 is a FLAG-tagged version under control of the Rosa26 promoter, this cannot be explained by a positive feedback response. Indeed, both Hedgehog-stimulated and unstimulated fractions contain comparable levels of GLI proteins. A similar enrichment of these complexes was noted in differentiating ES cells after LIF withdrawal (Fig 3.4B). This suggests that Hedgehog signaling promotes the formation of GLI1-NANOG complexes in ES cells.

3.7 NANOG REPRESSES GLI1-MEDIATED TRANSCRIPTION IN DIFFERENTIATING ES CELLS

The previous experimental results suggested that NANOG acts as a transcriptional repressor of GLI1-mediated transcription. These conclusions were based on experiments

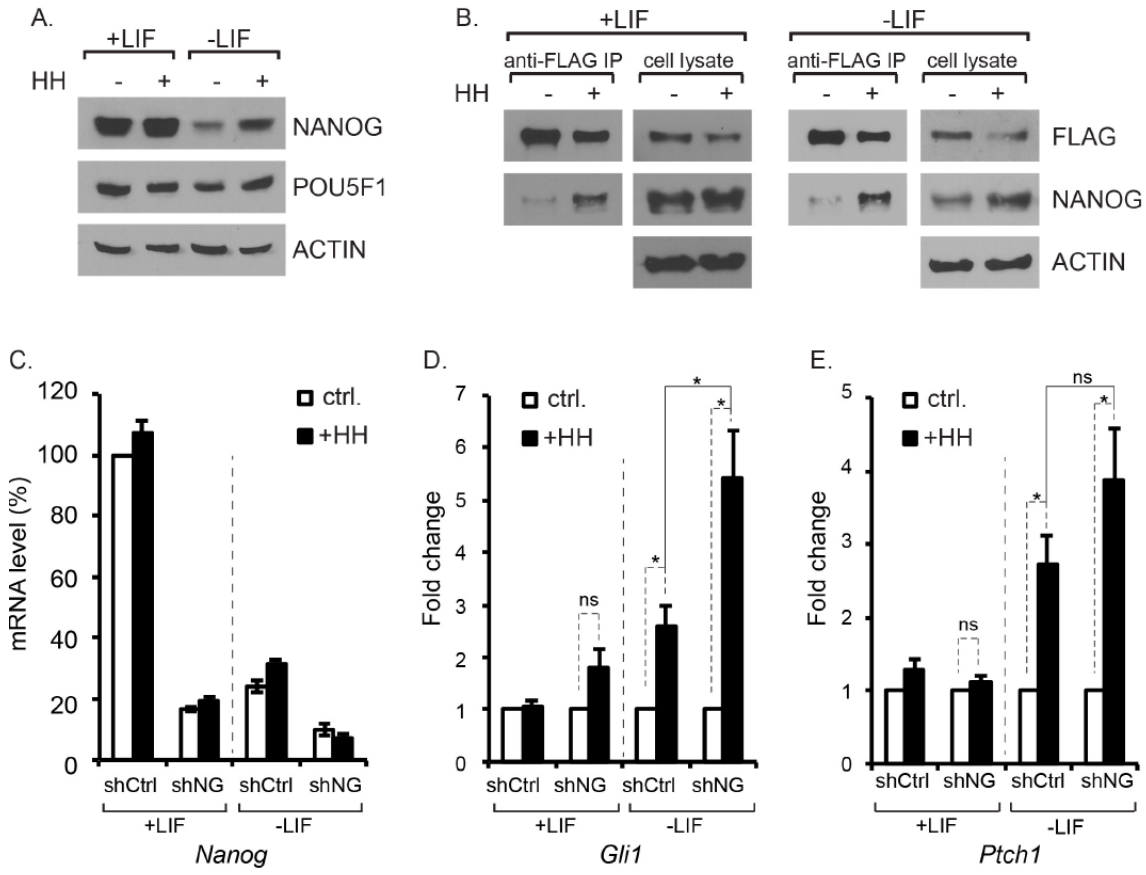

Figure 3.4 NANOG represses Hedgehog signaling target genes in differentiating ES cells

A: NANOG protein level was upregulated by purmorphamine (+HH) treatment in the absence of LIF, but not in the presence of LIF. B: Anti-FLAG immunoprecipitations show significantly more NANOG protein binding to $GLI1^{FLAG}$ upon PM treatment. C, D: Induction of *Gli1* and *Ptch1* mRNA levels in response to PM treatments in the condition of *Nanog* knockdown. RT Q-PCR analysis showed in the absence of LIF, that *Gli1* and *Ptch1* mRNA levels are upregulated 2-3 folds upon PM treatment, but not in the presence of LIF (compare "shCtrl-LIF" and "shCtrl+LIF"). Further knockdown of Nanog by lentiviral shRNA enhances the induction of *Gli1* and *Ptch1* in the absence of LIF (compare "shCtrl-LIF" and "shNG-LIF"), but not in the presence of LIF. Statistical significance was measured by a two-tailed, paired T-test from three independent experiments, *:$p<0.05$, ns: not significant, $0.1>p>0.05$.

that overexpressed NANOG. If NANOG represses GLI1-mediated transcription in ES cells, then reducing NANOG levels should elevate Hedgehog-mediated transcriptional responses. To test this, we infected ES cells with a lentiviral Nanog shRNA construct (shNG). Under pluripotent conditions with LIF, shNG infection resulted in an average 80% reduction in Nanog mRNA level (Fig 3.4C). As expected, Nanog levels are strongly downregulated upon LIF withdrawal, and were reduced a further 10% in shNG infected cells (Fig 3.4C). To determine if the reduced levels of NANOG levels affected Hedgehog-mediated transcription, we examined the expression of the pathway targets *Gli1* and *Ptch1*, which are direct Hedgehog pathway transcriptional targets in a variety of different tissue types. Under normal ES cells conditions, we did not observe a difference in *Gli1* or *Ptch1* in Hedgehog stimulated cells. However, when ES cells were cultured under conditions where LIF was withdrawn, there were significant upregulations of *Gli1* ($p=0.0282$) and *Ptch1* ($p=0.0232$) upon Hedgehog stimulation. Similarly, in shNG-infected cells, there was no significant Hedgehog-mediated upregulation of *Gli1* or *Ptch1* in the presence of LIF while upon LIF-withdrawal, *Gli1* and *Ptch1* were upregulated (Fig 3.4D, E). These experiments indicated that ES cells that begin to differentiate become Hedgehog-responsive. Interestingly, shNG-infected cells had significantly stronger levels of *Gli1*-induction compared controls in the differentiating ES cells ($p=0.036$, Fig 3.4D). The levels of *Ptch1*, while elevated, were not significantly different than controls ($p=0.1242$, Fig 3.4E). Together, these results are consistent with a model where NANOG reduces Hedgehog responses by binding to and repressing GLI proteins.

3.8 DISCUSSION

In this study, we have identified a previously unknown protein-protein interaction

between NANOG and GLI proteins. We show that expression of NANOG inhibits the ability of GLI proteins to activate transcriptional reporters or endogenous target genes. This suggests that NANOG may repress the transcriptional activity of GLI proteins. Consistent with this, reduced levels of NANOG levels increases the level of transcriptional response to HH signaling in differentiating ES cells. HH signaling also promotes the upregulation of NANOG, thereby generating a negative feedback loop. As NANOG is expressed in a variety of different stem cell populations, this interaction suggests a stem-cell-specific mechanism for dampening transcriptional responses to HH signaling.

We used a mass-spectroscopy-based approach to identify proteins specifically enriched in cells expressing FLAG-tagged GLI3-R. In addition to identifying SUFU and 14-3-3, previously characterized GLI binding proteins, we identified NANOG and its co-factor DAX1 as well as approximately 20 other proteins. While this approach was clearly successful at identifying GLI-associated proteins, the proteins were detected at low levels. GLI3 (the bait protein), had an average of 85 spectral counts per experiment while most interacting proteins, including SUFU, 14-3-3, NANOG and DAX1 had only a few spectral counts per experiment and were not identified in all three experimental replicates (Table 1). Future strategies for improving the number of spectral counts would increase the signal and therefore improve the utility of this approach for identifying GLI co-factors.

While HH signaling has well-established roles in adult stem cell homeostasis, it is not clear if HH signaling is active in mouse ES cells. It is not essential for mouse ES cell survival and ES cells lacking the essential receptor SMOOTHENED are capable of contributing to a range of different tissues in chimeric mice (Wijgerde et al., 2002). For these reasons, the interactions we report may be more applicable to understanding HH-

regulated transcription in adult stem cells. Interestingly, a GLI1-NANOG signaling has been described in an important positive feedback loop in neural stem cells. In particular, GLI1 acts as a transcriptional activator of NANOG in glioma stem cells and cerebellar neurospheres (Po et al 2010; Zbinden et al., 2010). Consistent with this, we find that HH signaling upregulates NANOG protein in differentiating ES cells. It is possible that this occurs through transcriptional activation although the increased levels of Nanog were not statistically significant. Regardless of the mechanism, HH signaling ultimately causes increased amounts of NANOG protein, thereby acting as a positive regulator. Upregulated NANOG levels would then be able to form complexes with GLI-A proteins that would inhibit subsequent GLI activation. In doing so, this is acting as a negative feedback loop (Fig 3.5).

This negative feedback loop contrasts with the positive feedback loops by which GLI and NANOG have been proposed to function in human glioblastoma stem cells and cerebellar neurospheres (Po et al., 2010; Zbinden et al., 2010). A possible explanation for this apparent difference might be that NANOG does not appear to completely block HH signaling. It is possible that the upregulation of GLI signaling observed in these other systems might be indicative of reduced but still active levels of GLI transcription. The over-activation of HH signaling in neural stem cells by *Ptch1* deletion results in reduced numbers of differentiated neuronal progeny and increased numbers of neuronal stem cells (Ferent et al., 2014). Since neuronal stem cells also require HH signaling to generate differentiated neuronal progeny (reviewed in Petrova and Joyner 2014), the levels of HH signaling perceived by neuronal stem cells must be tightly regulated to ensure a balance between stem cell renewal and differentiation. By regulating GLI levels, NANOG could provide another level of negative feedback to regulate the transcriptional response to HH signaling in ES cells.

Two different categories of models could explain how NANOG represses activation of GLI target genes. NANOG could sequester GLI proteins, preventing them from binding to their DNA target sequences. Alternatively, NANOG might associate with GLI proteins as they bind to DNA and subsequently recruit repressor proteins. We favor the latter class of models because the C-terminal region of NANOG robustly binds GLI1 but does not inhibit GLI target gene activation (Fig 3.3A-C). Consistent with the ability to recruit repressors, NANOG has previously been shown to associate with several repressor complexes that also contain OCT4 (Liang et al., 2008). Because GLI proteins do not include OCT4 (Fig 3.1A), the NANOG::GLI1 complex could potentially include distinct as well as common repressors. One possible candidate for this complex is DAX1, which was also identified as a GLI3-interacting protein in the mass spectrometry dataset (Table 3.1). DAX1 is a transcriptional co-repressor in several contexts, including ES cells (Li et al., 2011; Uranishi et al., 2013; Sun et al 2009). Like NANOG, DAX1 is a core member of the ES cell pluripotency network and has previously been identified as factor binding NANOG (Kim et al., 2008; Wang et al., 2006; Zhang et al., 2014; Wang et al., 2008; van den Berg et al., 2010). In addition to binding GLI1, NANOG also binds to a truncated, repressor-specific form of GLI3 (Fig 3.1). While assays for GLI transcriptional activation are straightforward, genetic approaches are currently the only meaningful way of determining loss of GLI-R without concomitant GLI activation. In future studies, it will be interesting to determine if NANOG binding also affects GLI-R activity.

NANOG has previously been shown to interact with Smad1, a transcriptional mediator of BMP signaling. Similar to what we have shown in this study, NANOG binds to Smad1 and inhibits BMP-mediated responses that would normally drive ES cells to differentiate (Suzuki et al., 2006). NANOG has also been shown to prevent NFκB induced differentiation by binding to NFκB family transcription factors (Torres and Watt,

2008). Together with our results, these studies indicate a common mechanism by which NANOG inhibits transcription. Interestingly, the C-terminal half of NANOG that binds GLI proteins also mediates the interaction between NANOG and the NFκB family transcription factor REL-A (Torres and Watt, 2008). It remains to be determined if NANOG binds to these different proteins through an adapter protein or via direct interactions, perhaps through a common protein motif.

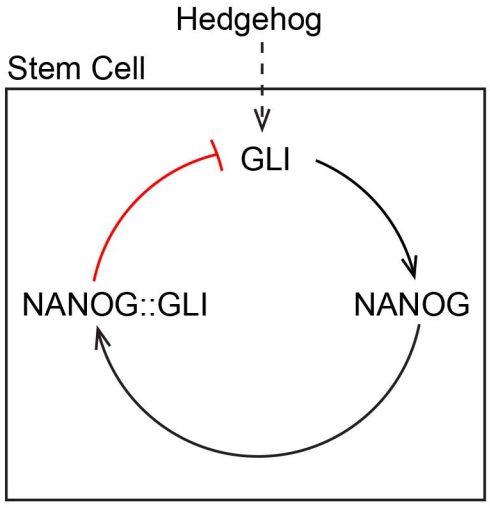

Figure 3.5 NANOG acts as in a negative feedback loop for HH signaling in ES cells

Chapter 4: Future directions

4.1 Explore GLI-NANOG interaction in adult stem cells

In Chapter 3, I uncovered the GLI-NANOG interaction in ES cells and further demonstrated that NANOG is able to repress GLI-mediated transcriptional activation. Many studies suggest that Shh also regulates adult tissue homeostasis through regulating adult stem cell populations within these tissues (reviewed in Alvarez-Buylla and Ihrie, 2014; Beachy et al. 2004; Jiang and Hui, 2008; Petrova and Joyner, 2014). However, how Shh-mediated regulation occurs at molecular level in these adult stem cell populations is still unknown. One of the interesting future directions is to determine if the GLI-NANOG interaction also exists in adult stem cells and what is the biological significance of this interaction.

Neural stem cells are quiescent stem cells mainly located in the subventricular zone (SVZ) of the lateral ventricle and in the subgranular zone (SGZ) of the hippocampal dentate gyrus (reviewed in Fuentealba et al. 2012). During neurogenesis, NSCs give rise to short-lived transit-amplifying cells to generate new neurons in the adult brain. Hedgehog signaling has extensive roles in the regulation of this process (reviewed in Petrova and Joyner, 2014; Traiffort et al. 2010). Shh signaling is required for the establishment of the NSCs in both the SVZ and SGZ (Balordi and Fishell, 2007; Han et al. 2008). Once the SVZ and SGZ are formed, Shh signaling is continuously required for their maintenance (Ahn and Joyner, 2005). During this process, Shh signaling need be maintained at proper level: gain or loss of Shh signaling enhances or inhibits proliferation of NSCs, respectively (reviewed in Petrova and Joyner, 2014). However, the mechanisms of maintaining the appropriate Shh signaling level in NSCs during neurogenesis are still

unknown. In NSCs, Hedgehog signaling directly activates *Nanog* transcription (Po et al. 2010), and as I showed in Chapter 3, NANOG is able to represses Hedgehog signaling by interfering with GLI proteins. These results leads to the hypothesis that Hedgehog signaling and NANOG form a self-regulatory feedback loop in NSCs to maintain correct level of Hedgehog signaling during neurogenesis. In future experiments, this could be addressed using following strategy.

First, does GLI1 physically interacts NANOG in NSCs? This could be addresses by using FACS sorting to purify both NANOG positive NSCs and NANOG negative NSCs from SVZ, and determine if Shh target genes are highly expressing compared to NANOG negative NSCs. I will use immunostaining to determine if GLI and NANOG proteins are co-localized in NSCs and use co-immunoprecipitation to verify the GLI-NANOG physical interactions.

Second, does NANOG repress Shh target genes in NSCs? In Chapter 3, I showed that NANOG represses GLI-mediated transcriptional activation to inhibit Shh-induced differentiation. This could be addressed by performing a tissue-specific knockout of *Nanog* in NSCs. If the transcriptions of Shh target genes such as GLI1 is altered due to decrease of NANOG, follow up studies could then use NANOG ChIP to determine if the changes of transcriptions of Shh target genes are directly due to loss of NANOG at promoter/enhancer regions of those genes. Finally, this could be addressed by testing Shh gain or loss of function in the presence and absence of NANOG in NSCs, to determine if NANOG interferes with Hedgehog signaling during neurogenesis.

4.2 MASS SPECTROMETRY ANALYSIS OF GLI PROTEIN COMPLEXES DURING LIMB DEVELOPMENT

The function of GLI has been extensively studied as its key role in the Shh signaling pathway. However, aside from neural-specific SoxB1 proteins (Oosterveen et al., 2012; Peterson et al., 2012), little is known about the tissue-specific transcriptional co-factors involved in GLI-mediated regulations at different embryonic tissues. Previous studies attempting to identify GLI associated co-factors were conducted with established stable cell lines that express epitope-tagged GLI (Asaoka et al., 2010; Jagani et al., 2010). Considering the tissue specification and organ patterning directed by Shh signaling is a very complicated process that involves proliferation and differentiation of different types of cells, the generic GLI-repressor complex might not exist in those highly specified homogeneous cell lines. To circumvent this, it is ideal to purify GLI protein complexes from embryonic tissues where Shh plays important regulatory roles.

Affinity purification and mass spectrometry are widely used approaches to identify new protein co-factors in existent protein complexes. However, there are no publications describing their successful applications in embryonic tissues. Two conditional mouse lines harboring FLAG-GLI1 and FLAG-GLI3R were previously established by our lab and successfully used for GLI ChIPs within embryonic tissues (Vokes et al. 2007, 2008). To identify the GLI co-factors in more relevant contexts, I propose crossing the conditional FLAG-GLI1 and FLAG-GLI3R lines with PrxCre line to specifically express FLAG-tagged GLI activator (GLI1) and repressor (GLI3R) in the limb buds respectively. This will allow us to study both GLI activator and repressor complexes systematically in embryonic limb buds.

Chapter 5: Materials and Methods

Experiments involving mice were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin). The compound crosses of mice used to determine genetic interactions were compared with littermate controls. The genomic coordinates in this study are reported in the mm10 build 38 genomic assembly.

5.1 Generation of Mouse strains

The *GRE1LacZ* transgenic line, officially named Tg(Rr26-lacZ)438Svok (MGI:5052053), was generated by pronuclear injection using the previously described enhancer reporter construct containing the 438-bp GLI binding region (chr2:113640843-113641280) (Vokes et al., 2008). The BAC transgenic constructs were generated using the Quick&Easy BAC Modification Kit (Gene Bridges) to modify a previously generated BAC containing *LacZ* within the *Gremlin* transcript (Zuniga et al., 2004). The homology arms for both targeting vectors were chr2:113,640,295-113,640,842 and chr2:113,641,281-113,641,757. After targeting, the FRT-flanked neomycin- resistance cassette was removed with a heat shock inducible FlpE construct (Gene Bridges), leaving a 69-bp FRT site and linker sequence precisely in place of the GLI CRM (chr2113640843-113641280). The GLI binding sites within the CRM were mutatedfrom TAGGTGGTC (chr2:113641085-113641088) to TACCACGTC and CACCTCCCA (chr2:113641174-113641177) to CACGTGGCA; the mutated CRM was flanked by a 5'EcoR1 site and a 3' 70bp linker sequence that included the FRT site. The official name for the $Gremlin^{GRE1}$ allele is Rr26<tm1Svok> (MGI:5486166). This allele results in the replacement of the438-bp CRM sequence with an 89-bp sequence containing a single LoxP scar. A 7.5kb genomic fragment containing the *Gremlin* CRM (chr2:113,637,382-

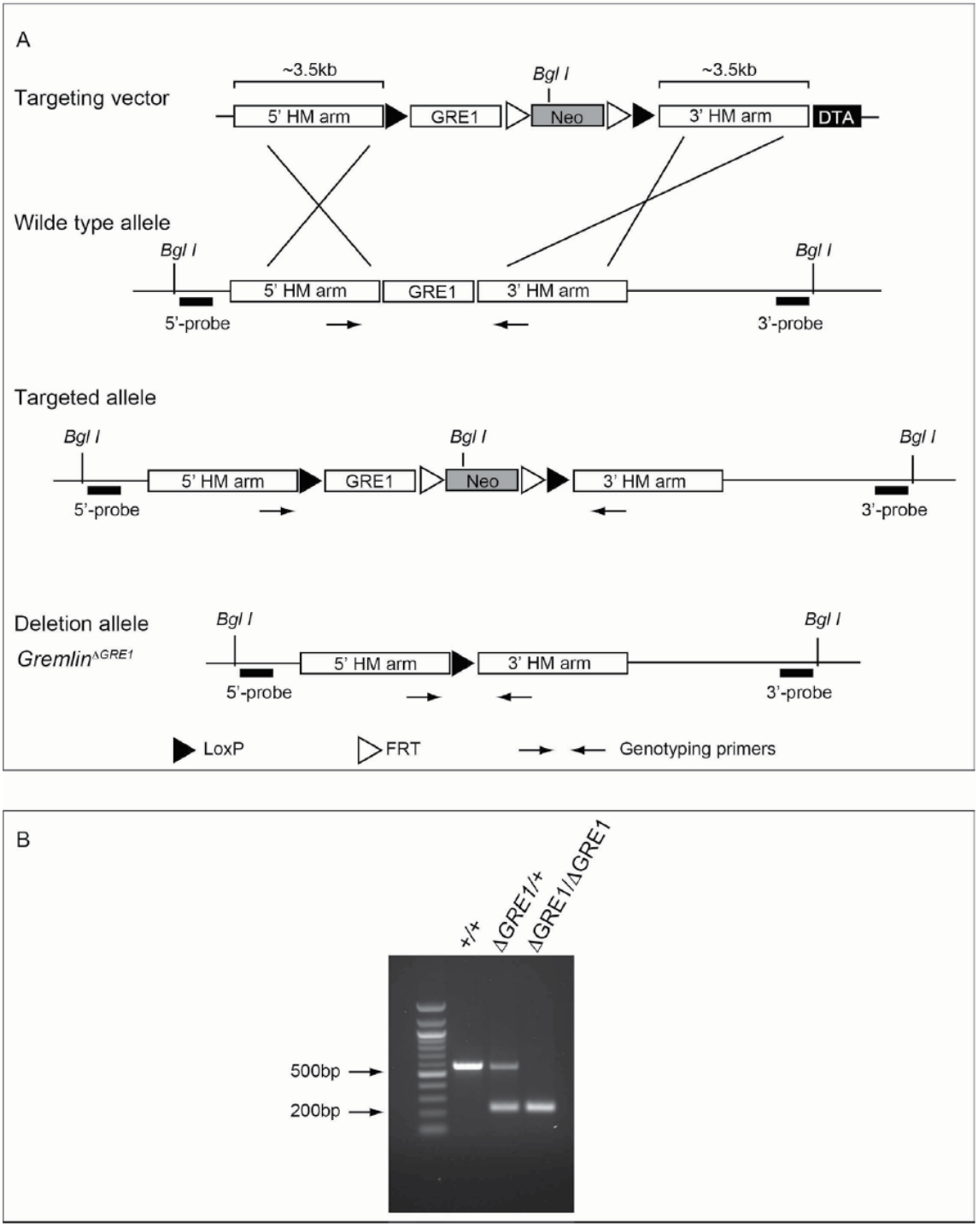

Figure 5.1 Generation of mice with a deletion of GRE1

113,644,893) was digested from BAC #RP23-113H17 with *Xma1* and *Kpn1* and cloned into a pBluescript upstream of a diphtheria toxin A (DTA) negative selection cassette (Fig 5.1A). A single loxP site and then an FRT-Neo-FRT-LoxP cassette were inserted immediately upstream and downstream, respectively, of the CRM (chr2:113640843-113641280). The targeting vector was linearized with KpnI and electroporated into AV3 ES cells (obtained from Dr. Andy McMahon's laboratory). Approximately 200 colonies were screened by Southern blot. DNA was digested with *Bgl I* and hybridized with a 5' probe corresponding to chr2:113636128-113636494 and a 3' probe corresponding mm10| chr2:113,648,199- 113,648,558. We identified two correctly targeted colonies by Southern blot, which were used to generate chimeric mice. Germline-transmitting chimeras were crossed with a Cre deleting strain, *Sox2Cre,* to generate a deletion allele, *Gremlin*$^{\Delta GRE1}$ (MGI:5486166). Mice that were wild- type, heterozygous and homozygous null mice were determined by genotyping with primers flanking the CRM (indicated by arrows underneath targeting constructs) that amplify a 582bp fragment in the wild-type allele and a 210bp fragment in the deletion allele (Fig 5.1B). The sequences of the primers are: 5'-GCTAAACACAAAGAACTTTTAATGG-3' and 5'- GCAGCAGCAGT A TTTTTCAGA-3'.

5.2 EMBRYONIC MANIPULATION

When applicable, limbs were cultured for 15 hours in 10 M Cyclopamine dissolved in absolute ethanol (Toronto Research) or 0.125% absolute ethanol for controls. After incubation, limb buds were separated from adjacent tissues and processed for qRT-PCR, β-galactosidase staining, or *in situ* hybridization. All limb buds were assayed for β-galactosidase activity by staining overnight using established methods (Whiting et al.,

1991). Skeletal preparations were performed as described previously (Allen et al., 2011).

5.3 STATISTICS

Unless indicated otherwise, in Chapter 2, statistical significance was measured using Fisher's Exact Test with a two-tailed P-value. In Chapter 3, statistical significance was measured using paired T-test with a two-tailed P-value, from three independent experiments was used.

5.4 QUANTITATIVE RT-PCR

RNA was extracted from a single pair of E10.5 embryonic limb buds (Chapter 2), or different cell lines (Chapter 3) using the RNA-Aqueous 4-PCR kit (Ambion) and subsequently treated with DNAse1. cDNA was synthesized from 300ng of total RNA with random hexamers using SuperScript II (Invitrogen). qRT-PCR experiments were performed using Power SYBR Green (Applied Biosystems) on a Viia7 system (Applied Biosystems). Target gene expression was determined by amplifying with the following primer pairs:

Fmn1: F-GACGCCGCACCAACTTTATG,
R-GGCCTCTGACAGGGGTTTTT;
GAPDH: F-GGTGAAGGTCGGTGTGAACG,
R-CTCGCTCCTGGAAGATGGTG;
Gli1: F-CCCAGCTCGCTCCGCAAACA,
R-CTGCTGCGGCATGGCACTCT;
Gremlin: F-ACTCGTCCACAGCGAAGAAC,

R-TCATTGTGCTGAGCCTTGTC;

Jag1: F-GTGCTACAATCGTGCCAGTG,

R-GGGGACCACAGACGTTAGAA;

LacZ: F-GGGCCGCAAGAAAACTATCC,

R-TCTGACAATGGCAGATCCCA;

Shh: F-TCTCGAGACCCAACTCCGAT,

R-GACTTGTCTCCGATCCCCAC;

Nanog: F-AGGGTCTGCTACTGAGATGCTCTG,

R-CAACCACTGGTTTTTCTGCCACCG

The GAPDH primers are widely used and the *LacZ* primers were previously described (Jeong et al., 2002). Unless specified otherwise, gene expression levels were normalized to GAPDH.

5.5 TISSUE CULTURE AND CELL LINES

NIH3T3 and HEK 293Tcells were cultured with 10% calf serum(CS) in DMEM. P19 cells were cultured wtih 2.5% fetal bovine serum (FBS) and 7.5% CS in α MEME (Sigma, #M8042). ES cell lines containing a Tamoxifen inducible Cre (CreER) and FLAG-tagged Gli1 or Gli3T (Vokes et al., 2007) were grown on MEF feeder cells. Expression of FLAG-tagged Gli1 and Gli3T was induced by adding 1mM 4OH-Tamoxifen (Sigma, #H7904) for at least 48 hours. The feeder free J1 ES cells (ATCC, cat# SCRC 1010.) were cultured on gelatinized plates. All ES cells were cultured in media containing 15% FBS DMEM media with leukemia inhibitory factor (LIF) at final concentration of 1000unit/ml in growth media except for LIF removal experiments.

5.6 shRNA lentivirus infection

1200ng of Nanog shRNA lentiviral plasmid (shNG; Sigma Mission RNAi # TRCN0000075333) or control (shCtrl; pLKO.1-puro vector containing 1.9Kb of inert DNA) was co-transfected with400ng of VSVG and 800ng of Δ8.9 into HEK293T cells in 6-well plates using Lipofectamine 2000 (Life Technologies). After one day, the media was changed to ES cell media without LIF in order to obtain LIF-free supernatant for ES-cell infection. After an additional 24 hours, the supernatant containing the viruses was harvested. Immediately before infection, the undiluted supernatant was mixed with polybrene (Sigma, #107689) to a final concentration of 4ug/ml and then mixed with 5x105 of resuspended J1 ES cells (ATCC, Cat# SCRC 1010). The ES cells were then incubated overnight before providing fresh media (day 2). The ES cells were split day three into ES cell media containing 5 μg/ml puromycin and treated with 5 μM purmorphamine or 0.02% DMSO as control for 2 days.

5.7 Immunoprecipitation and Mass spectrometry

$3x10^8$ ES cells expressing FLAG-tagged GLI1/3R or control cells were harvested by using cell scrapers in cold DPBS. Cells were spun down at 300g for 5 min and 1 ml of Lysis buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.5M NaCl, 0.2% Triton X-100, 10 mM KCl, 10% glycerol, add 0.5 mM DTT and Protease Inhibitor Cocktail Tablet, Roche) was per 0.3 ml of cell pellet. The cells were incubated with the lysis buffer at 4°C for 30min and spun down at 20000xg for 30min. 1 ml of supernatant was transferred into fresh tube and 0.3 volume of Buffer A (10 mM Hepes, pH7.9, 1.5 mM MgCl2, 10 mM KCl, 10% glycerol) was added to dilute the salt concentration of cell lysate to a final

concentration of about 0.3M. 50ul of Anti-FLAG M2 Agarose (Sigma A2220) was mixed with 1.3 ml of cell lysate and rotated at 4°C for 2 hours then washed three times with Washing Buffer (10mM HEPES, pH 7.9, 1.5mM MgCl2, 300mM NaCl, 10mM KCl, 0.2% TritonX-100, Complete Mini Protease Inhibitor Cocktail Tablet (Roche)) and then with Elution Base Buffer (10mM HEPES, pH7.9, 0.1M NaCl, 1.5mM MgCl2, 0.05% TritonX-100, Complete Mini Protease Inhibitor Cocktail Tablet (Roche), no FLAG peptides) once. The solution was spun down at 2500xg for 30sec. 30 ul of Complete Elution Buffer (200ug/ml 3xFLAG peptides, Sigma, #F4799 in Elution Base Buffer) was added and the mixture was incubated at 4°C for 30min. The supernatant was harvested by spinning at 2500xg for 30min. 20ul protein elutions were loaded on a 4-20% gradient SDS-PAGE gel (Biorad, #4561093) and minimally resolved by electrophoresis for 10 minute at 120V. The gel was subsequently stained with Coomassie Blue for 1 hour and destained for 30 minuntes. The stained part of gel containing proteins was excised and proteins were digested with trypsin in gel. Peptides were sequenced on a liquid chromatography tandem mass spectrometry (LC-MS). Peptide identification was performed using Scaffold software.

5.8 WESTERN BLOTS

Protein samples were resolved by 9% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, #10402468). Membranes were blocked with 10% non-fat milk in TBST buffer for 30min and incubated with primary antibodies: anti-FLAG antibody (Sigma, FLAG M2 antibody, F1804,1:4000), anti-HA (Thermo Scientific, 26183, 1:4000), anti-Nanog (Calbiochem, SC1000,1:2000), anti-Actin (Sigma, A2066, 1:2000), anti-OCT4 (Santa Cruz, SC5279,1:1000), in 3% non-fat milk at 4°C

overnight. After washing with TBST for 5min, membranes were then incubated with secondary antibodies: HRP-conjugated Rabbit-anti-Mouse secondary antibody (Jackson ImmunoResearch, 1:5000), HRP-conjugated Donkey-anti-Rabbit secondary antibody (Jackson ImmunoResearch, 1:5000), at room temperature for 1 hour. After washing with TBST three times, membranes were developed by using ECL Prime Western Blotting Detection Reagent (GE Healthcare, #RPN2232) and visualized by exposure to CL-XPosure films (Thermo Scientific, #34091).

5.9 LUCIFERASE ASSAY

NIH3T3 cells or P19 cells were seeded at 0.5x105/well in 24-well plates and co-transfected using Lipofectamine 2000 (Life Technologies, #11668019) with 300ng of luciferase reporter plasmid ptcΔ136-pGL3 (Nybakken et al., 2005), 100ng of pCIG-GLI1 (Vokes et al., 2007), 200ng of pSV-β-galactosidase expression plasmid (Nybakken et al., 2005), and 0-100ng NANOG expression constructs. For the Luciferase assay in NIH3T3 cells, media was changed to 0.5% serum 24 hours after transfection. The cells were harvested and assayed for activity using the One-Glo Luciferase Assay Kit (Promega, #E6120). In P19 cells, the luciferase assay was performed 2 days after transfection. All luciferase activities were normalized with beta-galatcosidase activities by BetaFluor β-gal Assay Kit (Merck #70979).

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